UNIVERSITY OF ALBERTA

# PROTEIN METABOLISM DURING CATCH-UP GROWTH FOLLOWING ESCHERICHIA COLI INFECTION IN WEANLING RATS

BY

SUSAN ELIZABETH COLLINS SAMUELS

©

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Susan Samuels

PERMANANT ADDRESS:

95 Kimbark Boulevard

Toronto, Ontario

M5N 2Y3 CANADA

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*It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us, we were all going to Heaven, we were all going the other way...*

*It is a far, far better thing I do than I have ever done;...*

Charles Dickens
A Tale of Two Cities

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **PROTEIN METABOLISM DURING CATCH-UP GROWTH FOLLOWING ESCHERICHIA COLI INFECTION IN WEANLING RATS.**

V. E. Baracos (Supervisor)

M. T. Clandinin

M. I. McBurney

D. J. Millward

J. R. Thompson

27 December 1992

## ABSTRACT

The objective of this research was to study whole body and tissue protein metabolism during catch-up growth following infection in weanling rats. E. coli peritonitis proved to be a simple and practical infection model that produced a marked and reproducible effect on growth and tissue protein mass. Two days after infection, protein mass of gastrocnemius (-21%), tibialis anterior (-23%), skin (-16%), and gastrointestinal tract (-15%) was lower ($P<0.05$) in infected compared with control rats; liver protein mass was unchanged. Complete catch-up growth, as measured by body weight, nitrogen balance and tissue protein mass occurred within 3 weeks after infection. On a whole body basis this was achieved by augmenting nitrogen balance by increased apparent nitrogen digestibility, whilst nitrogen intake and urinary nitrogen excretion remained unchanged. These changes in nitrogen balance were most prominent day 4 to 14 after infection. To study catch-up growth in individual tissues, protein synthesis and degradation were measured in vivo, 4, 6, 8, 11 and 14 days after infection. In small intestine, catch-up growth was complete by day 4, suggesting a high priority for protein repletion in this tissue. Liver growth and protein synthesis were not affected by treatment at any time point. Over the study, muscle and skin growth were higher ($P<0.001$) in infected compared with control rats, but there were no changes in absolute rates of protein synthesis in these tissues. This occurred because RNA mass, in muscle, was maintained. Catch-up growth was achieved because absolute rates of protein degradation decreased ($P<0.05$). Results

obtained from *in vitro* studies in muscle supported this conclusion. This mechanism appears to be unique because accelerated rates of growth are usually associated with increased rates of protein synthesis and degradation.

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# I. INTRODUCTION

## A. GENERAL INTRODUCTION

Infectious diseases constitute the most common form of disease in humans and other animals (Beisel 1991). The young of a species are particularly susceptible to the detrimental effects of infection (Scrimshaw et al. 1968, Mata et al. 1977, Beisel 1984). A growing child typically experiences more than 100 separate episodes of infection before reaching adulthood, even in developed countries (Beisel 1991). Still greater problems occur in developing countries, where poverty, poor sanitary conditions, and malnutrition are commonplace (Mata et al. 1977, Beisel 1991). The immediate post-weaning period is a time of particular vulnerability due to the loss of maternal antibodies and increased exposure to pathogenic agents (Mata et al. 1977). In agriculture, production losses due to infection result in the loss of millions of dollars in revenues each year (Magwood 1983).

Poor growth is a characteristic response to infection (Rowland et al. 1977, Mata et al. 1977, Zumrawi et al. 1987, Tian and Baracos 1989). Figure I-1 demonstrates the cumulative effect of repeated infections on body weight in a Guatamalan infant; it should be noted that malnutrition is undoubtedly a compounding factor, which is known to increase the severity and frequency of infection (Scrimshaw et al. 1968, Beisel 1991). Infection has a multitude of metabolic effects on the whole body and individual systems of animals (Beisel 1984, 1991). A prominant feature of infection is poor or negative nitrogen balances; body protein stores such as skeletal muscle are likely the

most adversely affected (Beisel 1984, Rennie 1985). In severe or prolonged infections, the loss of body protein mass can itself be life threatening (Wilmore and Kinney 1981).

Obviously, the most direct solution to this problem would be to eliminate infectious diseases or to limit their growth depressing effects. Despite major scientific effort, infection persists and the resultant poor growth still occurs. Another approach would be to improve growth during the recovery phase following infection. That is, promote the restoration of body and tissue protein stores and accelerate the growth process. Improved growth could be attained through the development of nutritional or drug treatments. Unfortunately, the means of tissue protein repletion are not well understood. Furthermore, an understanding of the factors that lead to tissue repletion could be exploited in the catabolic phase of infection to prevent tissue protein losses. To proceed with this strategy, the growth process following infection must be explored, and the regulation of and mechanisms associated with tissue and whole body protein repletion researched.

This dissertation is an account of both observations made during recovery from infection and of some of the mechanisms involved during catch-up growth. Accordingly, an overview of the effects of infection, growth following periods of restricted growth, and some of the methodologies used to explore the mechanisms are presented.

## B. EFFECTS OF INFECTION ON GROWTH AND NITROGEN METABOLISM

Infection is characterized by fever, anorexia, lethargy, and body weight loss (Beisel 1984, 1991). Basal metabolic rate increases 10

to 20 percent in mild infections and up to 50 percent in severe infection (Long 1977, Wilmore and Kinney 1981). A characteristic metabolic response to infection in adults is body protein loss, which cannot be explained on the basis of reduced food intake alone (Beisel 1966, Powanda et al. 1972). In growing animals, there is a transition to poor or negative nitrogen balance (Beisel 1984). When an infection is severe, prolonged, or complicated by burn or trauma, a third of body protein mass may be lost (Wilmore and Kinney 1981). Even brief and mild infections cause net body protein catabolism (Beisel 1966, 1984, 1991; Beisel at al. 1967).

The metabolic responses to infection are exceedingly complex and involve a multitude of biochemical pathways. These alterations are undoubtly related in one way or another to the control or elimination of the invading organisms. Unfortunately, many of the metabolic responses of infection are not well understood. An account of the metabolic effects of infection is given by Beisel (1984, 1991). However, in order to inform the reader who is not familiar with this field, an overview of the more salient features of infection is presented.

Central to the body's response to infection is activation of the host defense system (Powanda et al. 1972, Beisel 1984, Rennie 1985). Mononuclear phagocytes are activated and release products, such as interleukin-1, which alert and coordinate the body's response to infection (Powanda and Beisel 1982, Beisel 1984, Baracos 1985). These signals activate the immune system, induce fever, and stimulate metabolic responses to infection (Beisel 1984, Baracos 1985).

The liver plays a central role in orchestrating and co-ordinating such metabolic changes. Accordingly, there is typically an increase in hepatic protein synthesis (Lust 1966, Powanda et al. 1972, Klasing and Austic 1984). These hepatic proteins are crucial to the host defense response, which include the acute phase proteins and proteins needed for complement and blood clotting systems (Beisel 1984). There is also a decrease in other hepatic cellular functions, which further implies the priority given to the synthesis of these vital proteins (Douglas and Shaw 1989).

The increased metabolic activities of the liver and host defense response require additional amino acids and energy. In the liver, glycogenolysis, glycolysis, gluconeogenesis, and glucose oxidation are stimulated (Beisel et al. 1984). Hepatic amino acid uptake is greatly increased; amino acids will be used for protein synthesis, gluconeogenesis, and oxidition for energy (Clowes et al. 1980a,b; Rosenblatt et al. 1983). The effects on lipid metabolism are much less stereotyped, and it is difficult to assess the importance of this fuel (Beisel 1984). It appears that fatty acid utilization is depressed, and thus this substrate is not a preferred fuels (Beisel 1984. Ketogenesis, interestingly, is inhibited and thus utilization of ketone bodies by liver and other tissues is minimal (Blackburn 1977, Beisel 1984).

To meet the energy and amino acid requirements of the host defense response, substrates must be supplied. Because food intake is depressed, these must come from body stores. Glycogen and especially protein stores are tapped and depleted (Beisel 1984). Fat depots may also become depleted during prolonged or chronic infections (Beisel 1984).

Skeletal muscle is the largest protein-containing tissue in the body, and provides a large readily available source of amino acids (Beisel 1984, Rennie 1985). Skeletal muscle metabolism during the acute stages of infection has been extensively studied. Amino acid release from skeletal muscle has been measured in septic humans and has been shown to increase substantially (O'Donnell et al. 1976, Clowes et al. 1980a,b). This loss of muscle protein mass may result from increased protein degradation, decreased synthesis, or both. In experimental animal models, skeletal muscle protein synthesis has been shown to decrease (Lust 1966, Fern et al. 1985, Tian and Baracos 1989) and degradation to increase (Goldberg et al. 1984, Fagan and Goldberg 1985, Hasselgren et al. 1986). There is little information about the contribution of other protein reservoirs. For example, epithelial tissues such as skin and gastrointestinal tract make a large daily contribution to whole body amino acid flux (Waterlow 1984), and thus might serve as important sources of amino acids during catabolic states.

Many hormones are also involved in the metabolic regulation of infection, including cortisol, insulin, glucagon, thyroid hormones, catecholamines, and growth hormone (Beisel 1984). The hormonal effects are extremely complex and interrelated, and depend on the severity and phase of infection. There are also a variety of non-hormonal factors, such as prostaglandins and cytokines, that may play an important regulatory role (Baracos et al. 1983).

Therefore, survival from infection depends not only on the ability of the immune system to eradicate invading micro-organisms, but on a series of co-ordinated hormonal, metabolic, and

physiological responses that maintain vital functions, such as fuel production and acute-phase protein synthesis. A failure to maintain these functions that are vital to the host defense response, results in overwhelming infection and death (Beisel 1984).

## C. CATCH-UP GROWTH

Poor nutrition and disease will often displace animals from their growth curves. When the growth impediment is removed, these animals will often display a rate of growth that is greater than expected for normal animals of the same age. This is defined as catch-up growth (Tanner 1986) (Figure I-2). In agriculture this is often called compensatory growth, i.e. the animal is compensating for lost growth time (Swatland 1984). (In zoology and pathology, however, there are many other kinds of compensatory growth; for example the removal of one kidney often stimulates the growth of the other.) For clarity, this accelerated growth phenomenon will be referred to as catch-up growth. The phenomenon of catch-up growth has been observed and studied under a variety of circumstances, including malnutrition, hypothyroidism, and growth hormone deficiency. Prader et al. (1963) discuss many examples of catch-up growth following nutrient deprivation and illness in children. Catch-up growth has been extensively studied in domestic animals following food and protein deprivation, although almost exclusively on a whole animal basis, and has been reviewed by Wilson and Osbourn (1960).

Catch-up growth following infection has been observed for centuries (Tanner 1986). Stoeller (cited by Tanner 1986) in his textbook on growth in 1729 noted:

> Frequently illnesses stop people growing, but if a feverish or long standing malady is properly overcome then people grow very much; so that as a rule those persons shooting up in height, who particularily in their childhood have been held in check by hot or cold fevers.... This is the basis of the well-known proverb "illness laid him low and stretched him out,".

More recently, catch-up growth has been noted in epidemiological and field studies (Scrimshaw et al. 1968, Duggan and Milner 1986). Other studies, however, indicate that children afflicted with repeated infections concomitant with poor nutrition never catch up (Scrimshaw et al. 1968). Unfortunately there is a lack of controlled scientific experimentation that addresses catch-up growth following infection.

Catch-up of whole body protein mass must occur by increasing nitrogen balance. Nitrogen balance may be augmented by: 1) increasing the amount of nitrogen available to the body, either by increasing nitrogen intake or by decreasing faecal nitrogen excretion or 2) by increasing the efficiency with which it is used, that is decrease urinary nitrogen excretion (Figure I-3). Increases in whole body nitrogen balance must also be accompanied by alterations in whole body protein synthesis and/or degradation. There is a lack of information regarding whole body nitrogen balance or protein turnover during catch-up growth following infection. Tomkins et al. (1983) measured whole body protein turnover in children recovering from infection, and found that protein synthesis and degradation were higher than in normal children (Golden et al. 1977). However, in this study, the recovering children were still in negative protein

balance. There is a pool of literature concerning catch-up growth following nutrient deprivation. During the rapid growth phase following recovery from malnutrition, increased whole body protein synthesis and degradation have been observed (Golden et al. 1977). However, the control subjects in this experiment were in a negative nitrogen balance. Increased food intake has been consistently observed (Wilson and Osbourn 1960, Widdowson and McCance 1963) and urinary nitrogen excretion has been shown to decrease (Whittemore et al. 1978). Apparent nitrogen digestibility does not appear to increase during realimentation (Wilson and Osbourn 1960, Whittemore et al. 1978). Thus, on a whole body basis, catch-up growth following nutrient deprivation appears to occur by increasing nitrogen intake and the efficiency with which nitrogen is utilized.

On an individual tissue basis, increased nitrogen balance must occur by increasing protein synthesis relative to degradation (Figure I-3). Again there is no information regarding tissue protein turnover during recovery from infection. Jepson et al. (1986) measured skeletal muscle protein turnover following repeated administration of endotoxin in rats. By the third day of endotoxin administration, rats began to recover from endotoxin, and rats had decreased fractional and absolute rates of protein synthesis and degradation. Millward et al. (1975) measured protein turnover in skeletal muscle from growing rats recovering from prolonged malnutrition. During catch-up growth, fractional and absolute rates of protein synthesis were higher than in age-matched controls. Fractional but not absolute rates of protein degradation were higher, although they did not measure protein turnover beyond 14 days into the recovery period.

Rates of protein synthesis are dependent upon the RNA mass and efficiency of protein synthesis (synthesis relative to RNA mass) (Millward et al. 1975). Skeletal muscle RNA mass has been shown to decrease during infection and endotoxin administration (Fong et al. 1991, Jepson et al. 1986). Catch-up growth following infection must also involve the restoration of RNA mass. If there are differences in protein synthesis during recovery from infection, then this will depend upon RNA mass and/or efficiency of protein synthesis. For example, during catch-up growth following prolonged malnutrition, RNA mass and efficiency of protein synthesis rapidly increase leading to increased rates of protein synthesis (Millward et al. 1975).

Despite the great impetus animals have to return to their normal growth curves, catch-up growth may fail. The ability to catch-up is dependent upon a number of factors, including the type, duration, and severity of the growth depressing stress, as well as factors such as age, sex, and species (Wilson and Osbourn 1960). There has been some experimentation suggesting the ability to catch up in skeletal muscle is dependent upon achieving a normal complement of DNA (Beermann 1983, Glore and Layman 1987).

## D. METHODOLOGY

To study the effects of infection on growth and protein metabolism requires a model that produces a sufficiently marked and reproducible effect, in which catch-up growth could be detected. There are a variety of experimental models, including acute and chronic infections, and the use of endotoxin (Wichterman et al. 1980). Endotoxin, while reproducible, is an acute treatment that

may not adequately reproduce the effects of infection. Many infections are either variable, unreproducible, or lethal. In our laboratory, Escherichia coli peritonitis has been successfully used in chicks and 200 g rats to cause a marked effect on growth and tissue protein mass without causing substantial mortality (Tian and Baracos 1989a,b). However, it has not been used in weanling rats nor to study catch-up growth.

To investigate the relative contributions of protein synthesis and degradation to augmented growth of tissues during recovery from infection requires the use of appropriate methods. Tissue protein turnover can be measured using a variety of methods. Protein synthesis is measured using isotopically labelled amino acids (Garlick et al. 1973, 1980b, Schaefer et al. 1986). When an isotope is injected it rapidly distributes into the free amino acid pools, from which it is incorporated into protein. Protein synthesis is determined from the amount of isotope incorporated into protein and the specific radioactivity of the precursor pool. The direct precursor for protein synthesis is aminoacyl-tRNA; however, this is difficult to measure (Sugden and Fuller 1991). Instead tissue or plasma free amino acid specific radioactivity is measured. If precursor specific radioactivity remains constant over the period of incorporation, protein synthesis can simply be calculated from the following equation:

$$\text{Protein synthesis} = \frac{\text{Protein-bound specific radioactivity}}{\text{Free amino acid specific radioactivity} \times t}$$

where t is the length of time of incorporation (McNurlan et al. 1979).

Protein synthesis can be measured in vivo or in vitro. In vitro methods while simple and inexpensive are only suitable for certain skeletal muscles (Sugden and Fuller 1991). The two commonly used in vivo methods are the continuous infusion and flooding-dose techniques. These methods are suitable for measuring protein synthesis in individual tissues.

Continuous infusion methods involve continuous infusion of an isotopically labelled amino acid (Sugden and Fuller 1991). The isotope may be included in the diet if it is continuously fed (Harney et al. 1976). To reach a rapid plateau specific activity, a bolus or priming dose of isotope is given at the start of infusion (Sugden and Fuller 1991). This method has been widely used in a variety of species including, rats, pigs, cattle, and humans (Garlick et al. 1973, 1976, Lobley et al. 1980, Waterlow 1967). The main criticism of this method lies with the identity of the precurser pool (Waterlow et al. 1978). The intracellular and plasma specific activities are sufficiently different to make estimates of protein synthesis based on either quite different (McNurlan et al. 1979, Pomposelli et al. 1985). In addition, recycling of tracer in tissues or proteins with a high rate of turnover may cause error (James et al. 1971).

The flooding-dose method involves the injection of a large amount of isotope along with a large amount of unlabelled amino acid, equivalent to several multiples of the whole body free amino acid pool (Garlick et al. 1980b). This results in rapid equilibration of specific activities in the aminoacyl-tRNA, intracellular, and plasma

pools (Garlick et al. 1980b, Sugden and Fuller 1991). Plasma and tissue specific activities remain almost constant during the period of incorporation (Garlick et al. 1980b, Reeds et al. 1982, Jepson et al. 1986, McAllister 1987). The short labelling period, 10 to 15 minutes, limits the recycling of amino acid in rapidly turning over proteins. The flooding-dose method could be used for any species, of any size, except for the large amount of frequently costly isotope that would be required in large animals. The reliability of the method also requires that the large amount of amino acid injected does not influence protein turnover. The most commonly used amino acid is phenylalanine (Phe). Phe is reasonably soluble, so a large dose may be given, and the free pool of Phe is small and easily flooded. It is also relatively inexpensive and is not known to have any direct effect on protein synthesis (Garlick et al. 1980b). The main drawback is that synthesis is only measured over a period of 10 to 15 minutes, which may not be representative of the daily overall rate of protein synthesis.

Tissue protein degradation can be measured using a variety of methods. In vitro methods involve determination of the rate of release of an amino acid, usually phenylalanine or tyrosine, from protein. This is the simplest method, but is only suitable for certain thin skeletal muscles (Sugden and Fuller 1991). To measure degradation directly, muscles are incubated in the presence of cycloheximide, which prevents reincorporation of amino acids (Tischler et al. 1982). Alternatively, degradation may be measured indirectly as the difference between synthesis and net release of amino acids (Tischler et al. 1982). In vivo methods can be used for

measures of degradation in any tissue, but are calculated as the difference between protein synthesis and net protein accumulation or growth (Sugden and Fuller 1991). The difficulty is that changes in growth are small and need to be measured over days, whereas synthesis is measured over minutes (Sugden and Fuller 1991). The calculation assumes that synthesis is constant over the time that growth is measured (Sugden and Fuller 1991). The protein mass of a tissue can only be measured once for each animal, therefore, growth is often assessed by serial slaughter of many animals prior to and following the measurement of synthesis (El Haj et al. 1986). Growth is then calculated by regression analysis of protein mass from each tissue against time (El Haj et al. 1986); this results in growth rates that are not based on an individual animal basis and thus statistical analyses cannot be performed. Another approach is to assess growth from daily body mass gain and final protein mass of each tissue (Attaix and Arnal 1987). Using this approach, growth and degradation can be calculated on an individual animal basis. Growth is calculated in a two stage process. First, protein mass from individual tissues is regressed against body mass for all animals on a given treatment (slope = $\Delta$ protein mass/ $\Delta$ body mass). Over the weight ranges studied, these regressions were linear. Body mass is then regressed against time for each animal (slope = $\Delta$ body mass/ $\Delta$ time). By multiplying these slopes, the protein mass gained per day for each rat can be obtained.

## E. OBJECTIVES

The objectives of this dissertation were:

1) To develop an infection model for use in weanling rats that

produces a marked and reproducible effect on whole body and tissue nitrogen status, by adapting a model of E. coli peritonitis used in chicks and 200 g rats.

2) To determine whether catch-up growth following infection occurs. To do so, body weight, food intake, nitrogen balance, and carcass and tissue protein mass will be monitored during the catabolic, catch-up, and post catch-up phases of infection.

3) To examine the mechanisms by which catch-up growth occurs, on a whole body and individual tissue basis. On a whole body basis this will be done through measurement of food intake, apparent nitrogen digestibility, and efficiency of nitrogen utilization. On an individual tissue basis this will be done through measurement of protein synthesis and degradation in skeletal muscle, skin, small intestine, and liver. Protein synthesis will be measured in vivo using a flooding dose of $^{3}$H-Phe. Protein degradation will be measured, in vivo, as the difference between synthesis and growth.

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Figure I-1 Effect of infection on growth of an infant

Chronological documation of the infections suffered by a single Guatemalan Indian infant. The cumulative effect of these infections on body weight (bottom curve) shown in comparison to the anticipated normal growth curve (top curve). Diagram from Mata et al. 1977.

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Figure I-2 Diagram of catch-up growth

'A' is a period of depressed growth.

'B' is a period of catch-up growth.

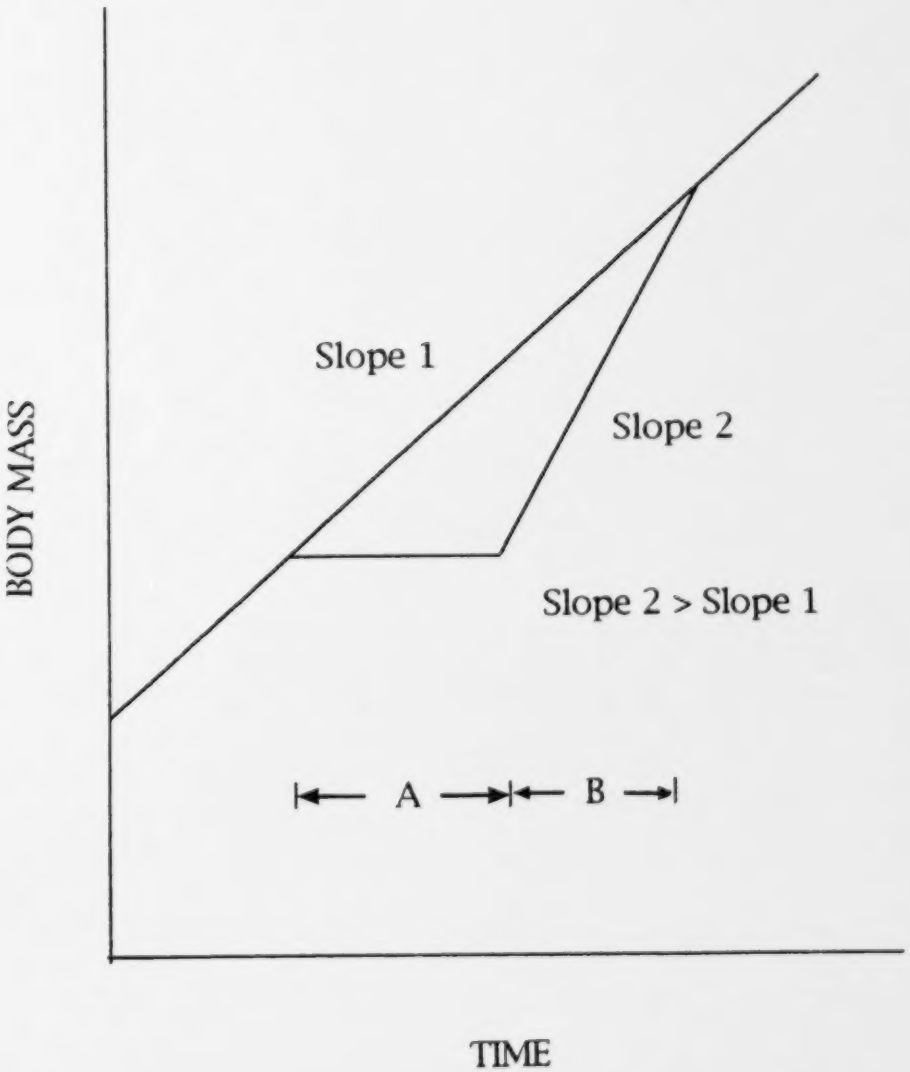
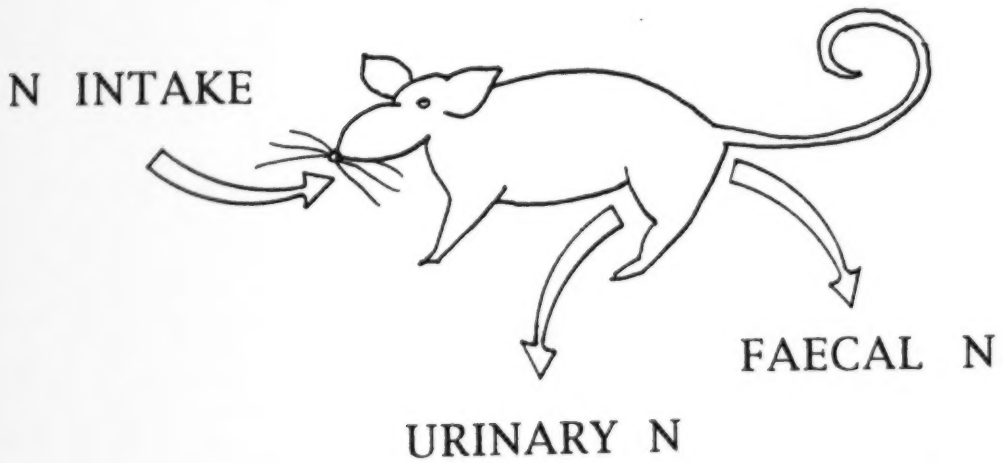
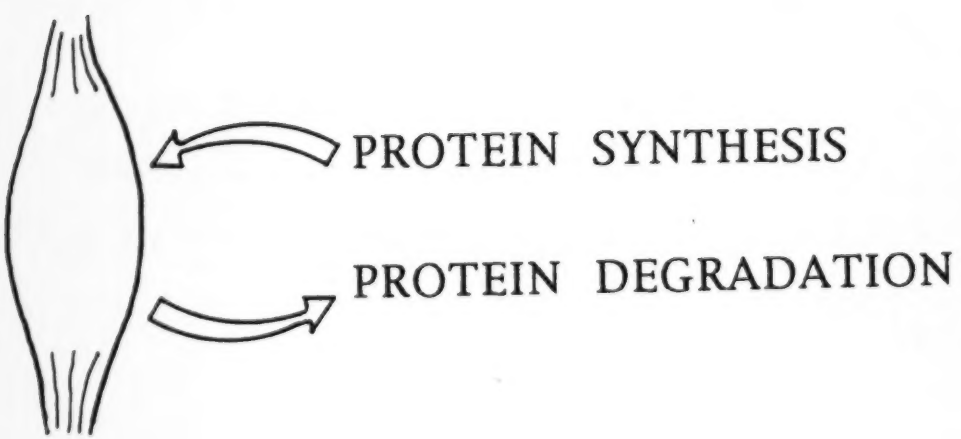


Figure I-3. Whole body or tissue protein or nitrogen balance




Whole body nitrogen (N) balance = N intake - urinary N - faecal N




Tissue protein or N balance = protein synthesis - protein degradation

# II. CATCH-UP GROWTH FOLLOWING *ESCHERICHIA COLI* INFECTION IN WEANLING RATS

## A. INTRODUCTION

Infection is detrimental to growth. Young animals are especially sensitive to the deleterious effects of infection (Beisel 1984, Tian and Baracos 1989a, Scrimshaw et al. 1968, Mata et al. 1977). Animals at weaning are particularly vulnerable because of an increased risk of exposure to pathogenic agents and a loss of maternal antibodies (Scrimshaw et al. 1968, Mata et al. 1977). For these reasons, infection is one of the most serious problems facing infants and children in developing countries (Scrimshaw et al. 1968). It is also a problem in agriculture, resulting in the loss of millions of dollars of revenue (Magwood 1983).

Central to the body's response to microbial invasion, is the activation of the host defense systems, and mobilization of body protein serves as a source of amino acids and energy to fuel the hyper-metabolic requirements of the defense response (Beisel 1984, Powanda et al. 1972, O'Donnell et al. 1976, Rennie 1985). Such mobilization can have an immense negative influence on body protein status (Tian and Baracos 1989a, Powanda et al. 1972, Wannemacher et al. 1975, Fern et al. 1985). Even when infections are brief, mild, or localized, disturbances of nitrogen economy can be detected (Beisel 1984, Garlick et al. 1980). In growing animals, infection is

[1] A version of this chapter has been published. Samuels, S. E., and V. E. Baracos. 1992. Metabolism 41: 208-215.

manifested by poor or negative nitrogen balance, and body and tissue protein wasting (Beisel 1984, Tian and Baracos 1989a, Powanda et al. 1972, Fern et al. 1985). Skeletal muscle is generally assumed to be a major site of protein catabolism (Rennie 1985, Wannemacher et al. 1975); less is known about the contribution by other tissues. For example, epithelial tissues such as skin and gastrointestinal tract make a large daily contribution to whole body amino acid flux (Waterlow 1984), and thus may serve as important sources of amino acids during catabolic states.

Complete recovery from infection involves replenishment of body stores, and return to the original growth trajectory. For this to occur, clearly growth must occur at a rate above that expected for normal healthy individuals at the same age; this is referred to as catch-up growth (Tanner 1986). Nitrogen balance must also be augmented; this may be accomplished by increasing nitrogen absorption, via increasing intake or digestibility, or by increasing the efficiency with which nitrogen is utilized. Catch-up after starvation or food restriction has been studied in diverse species (Wilson and Osbourn 1960, Widdowson and McCance 1963); intake, digestibility and/or efficiency of nitrogen utilization may all be involved in catch-up (Wilson and Osbourn 1960). Knowledge concerning the mechanisms of catch-up after infection is not available; this information would permit improved nutritional and metabolic strategies to promote catch-up growth.

There have been a few epidemiological and field studies where catch-up growth has been noted following infection (Scrimshaw et al. 1968). However, other studies indicate that children afflicted with

repeated infections concomitant with poor nutrition never catch up (Scrimshaw et al. 1968). Unfortunately, most experimental studies designed to examine the effects of infection on growth have focused only on changes in nitrogen metabolism in the catabolic phase (Tian and Baracos 1989a,b; Powanda et al. 1972, Fern et al. 1985). Experimental data, therefore, that address the effects of infection beyond the catabolic phase are needed.

To study the effects of infection on growth and protein metabolism, it is necessary to develop a suitable experimental model that will produce a sufficiently marked and reproducible effect on growth. We have adapted a model of E. coli peritonitis (Tian and Baracos 1989a,b) for use in weanling rats. Using this model we have made measurements of body weight, food intake, nitrogen balance, and carcass and tissue protein mass, during the catabolic, catch-up, and post catch-up phases of infection.

## B. METHODS

### Animals and Diet

All studies were carried out in compliance with the guidelines of the Canadian Council on Animal Care. Female Sprague-Dawley rats were weaned at 22 to 24 days of age and housed in individual metabolism cages. The animals were housed at 26°C and 60% humidity on a 12 hour light:dark cycle. The animals were fed a diet based on corn and soybean meal that met or exceeded the National Research Council (1978) recommendations for nutrients (Table II-1). Food and water were provided ad libitum. Animals were given at least 6 days to adjust to their new environment and diet before experimental treatments were imposed.

Experimental Infection

The E. coli were obtained from an outbreak of coliform septicaemia in turkeys and had been characterized in the chick in terms of dose, effect on growth, food intake, and protein metabolism (Tian and Baracos 1989a). Preliminary dose response experiments established that a dose of ~$10^9$ colony forming units (CFU)/kg body weight resulted in acute infection without causing a massive death rate. Refer to Appendix 2 for the preparation of E. coli. In Experiment (Expt) 1 and 2, rats were administered 6 x $10^9$ CFU/kg body weight, and in Expt 3, half of the infected rats were given 6 x $10^9$ CFU and half 3 x $10^9$ CFU/kg body weight. Rats were given a single intraperitoneal injection of E. coli in a volume of 0.5 mL of sterile phosphate buffered saline, or vehicle alone.

Experimental Design

The study was carried out in three experiments. A total of 99 rats (38 control and 61 infected) were initially used. Rats were randomly allocated to treatments on the day of infection. Thirty-six rats died as a result of the infection; only data from the 25 survivors were included in the analyses.

In Expt 1, data were obtained from 11 infected and 11 control rats. Variables measured were: body weight, food intake, faecal nitrogen, and urinary nitrogen; these were determined at the time points indicated in the tables and figures. These data were used to compute daily nitrogen balance, cumulative nitrogen balance, apparent nitrogen digestibility, and apparent efficiency of nitrogen utilization. Apparent nitrogen digestibility = (nitrogen intake -

faecal nitrogen excretion)/ nitrogen intake. Apparent efficiency of nitrogen utilization = (nitrogen balance/(nitrogen intake - faecal nitrogen excretion)). These experimental parameters were monitored until the cumulative nitrogen balance of infected rats was equal to that of control rats (day 18), and for 26 days thereafter.

Expt 2 was a repeat of Expt 1, but was terminated when the cumulative nitrogen balance of infected rats caught up to that of control rats (day 23). In Expt 2, data were obtained from 5 infected and 15 control rats. Measurements were made as for Expt 1.

Body and tissue nitrogen masses were also measured. Nitrogen analysis of carcass, skin, gastrointestinal tract, liver, gastrocnemius and tibialis anterior muscles were determined at days 18 and 44 (Expt 1), day 23 (Expt 2) to confirm catch-up. These time points were selected on the basis of the cumulative nitrogen balance data. A third experiment was conducted to determine the extent to which infection altered body and tissue protein masses. Data were obtained at two time points: 1) immediately prior to infection (day 0) (6 control rats) and 2) two days after infection when nitrogen balance was most supressed (9 infected and 6 control rats).

Sample Preparation

Urine was collected into containers containing 0.5 mL of 1 N $H_2SO_4$, weighed, and stored at -20°C until analyzed. Faeces were also collected and stored at -20°C until analyzed. All rats were killed by $CO_2$ asphyxiation. After the rats were killed, the contents of their gastrointestinal tracts (from distal oesophagus to the anus) were removed, emptied, and rinsed. Livers, right and left

gastrocnemius and tibialis anterior muscles were also dissected, weighed, and stored at -70°C. Gastrocnemius and tibialis muscles were used because they are of mixed fiber type, which is representative of the majority of skeletal muscles (Maltin et al. 1989). Animals were shaved of hair and then skinned. Skin and carcasses were autoclaved for 8 hours at 115°C, cooled, and homogenized with a polytron homogenizer. The entire homogenate was freeze dried and allowed to equilibrate to normal room humidity, then weighed and ground.

Analyses

Nitrogen contents of urine, faeces, tissues, and carcasses were analyzed by the Kjeldahl method (AOAC 1984). Duplicate subsamples of urine and faeces for each time period and each animal were used. Duplicate subsamples of carcass and skin were used for analysis. Whole gastrocnemius and tibialis anterior muscles, gastrointestinal tracts, and livers were used for nitrogen analysis. Protein masses of tissues and carcasses were determined by multiplying the nitrogen mass by 6.25. The DNA and RNA masses of gastrocnemius muscles were determined by the method of Munro and Fleck (1966).

Statistical Analysis

For each time point, the data were analyzed by a Student's t-test at the 0.05 level of significance. To determine if there were any differences in body weight between the control and infected rats between day 18 and 44, the data were analyzed using an analysis of variance with a repeated measures design (SAS 1988). To determine if

infected rats were growing and depositing nitrogen at a faster rate than control rats after the infection, regression analyses of body weight (day 1 to day 9) and cumulative nitrogen balance (day 2 to day 18) against day were performed for each animal (SAS 1988). Then, a Student's t-tests was used to assess if there were differences in slopes, i. e. regression lines, between treatments. To determine if nitrogen balance immediately after infection could be predicted by food intake, a regression analysis of nitrogen balance two days after infection against food intake one day after infection was performed (SAS 1988). Various parameters were assessed to determine which was the best indicator of the acute changes in nitrogen balance; food intake one day after infection proved to be the best indicator. Values are means ± SEM.

## C. RESULTS

### Pre-infection

There were no statistically significant treatment differences in body weight, food intake, faecal nitrogen excretion, urinary nitrogen excretion, nitrogen balance, apparent nitrogen digestiblity, and apparent efficiency of nitrogen utilization between control and infected rats for two to 6 days prior to treatments being imposed.

### Mortality and Morbidity

Of the 61 rats injected with *E. coli*, 36 died within 24 hours of being infected. There were no other mortalities. Infected rats showed lethargy, piloerection, and decreased grooming, for up to 24 hours after infection. The lower dose of bacteria in Expt 3 lowered

mortality; none of these rats died. Since there were no differences in body weight, food intake, or carcass and tissue protein masses after infection, between surviving rats given the two doses of E. coli, data were pooled. Infected rats in all three experiments were similar in terms of body weight losses and food intake.

The presence of bacteria was not detected when fluid from the peritoneal cavities of control and infected rats was cultured in tryptic soy broth after rats were killed on days 18, 23, or 44. In a pilot experiment, 16 rats were also tested for the presence of bacteria, either 7 or 14 days after infection; all eight tested positive on day 7, and four tested positive and four negative on day 14.

Catabolic Phase

Infection had marked effects on body weight, food intake, faecal nitrogen excretion, nitrogen balance, and apparent efficiency of nitrogen utilization (Tables II-1, II-2, II-3, II-5; Figure II-1; Appendix 1A, 1B, 1C, 1D, 1F). These effects were catabolic and lasted from one to four days after infection. Since Expt 1 and 2 were similar, only data from Expt 1 are presented (data from Expt 2 are shown in Appendix 1).

Body weight data was expressed as a percentage of each animal's day 0 body weight (mean ± SEM: 85.7 ± 1.3 g) (Figure II-1) (Appendix 1A & 1B). Body weights of infected rats were lower than healthy rats, one day after infection (-11.8%; $P<0.001$). Infected rats ate less than healthy rats, one day after infection (-65%; $P<0.001$), and continued to eat less than healthy rats until day 4 (Table II-2)

(Appendix 1C). Infected rats had lower daily nitrogen balances ($P<0.001$; -64.1%) (Table II-3) (Appendix 1D) and lower cumulative nitrogen balances ($P<0.001$) (Figure II-2) (Appendix 1E) than healthy rats, between day 0 and day 2; daily nitrogen balance was never negative. The apparent efficiency of nitrogen utilization was lower (-32%; $P<0.001$) in infected rats than in healthy rats, between day 0 and day 2 (77.4 ± 3.5% vs. 52.9 ± 4.1%) (Appendix 1F). There were no differences in apparent nitrogen digestibility during the first 2 days of infection (Table II-4) (Appendix 1G); infected rats, however, excreted less faecal nitrogen over this time period (-48.2%; $P<0.001$) (Table II-5) (Appendix 1H). There were no differences in urinary nitrogen excretion from day 0 to day 2, between control and infected rats (75 ± 12 vs. 71 ± 3 mg/rat/day) (Appendix 1I).

The results for Expt 2 were in principle the same as for Expt 1. The main difference was that rats on Expt 2 appeared to have been more severely affected by the infection. This was based on the fact that infected rats on Expt 2, compared to Expt 1, lost more weight (-1.8 g; $P>0.05$) one day after infection, ate less food (-4.7 g/rat/4 days; $P<0.05$) between day 0 and day 4, and had lower daily nitrogen balances (-33 mg/rat/day; $P<0.05$) between day 0 and day 4, and had lower apparent nitrogen digestibilities (78.1 vs. 70.2%; $P<0.05$) from day 0 to day 2.

In Expt 3, control rats showed net carcass and tissue protein gain over the first two days of study (Table II-6). By contrast, infected rats showed decreasing protein mass of gastrointestinal tract, and gastrocnemius and tibialis anterior muscles, over the same time interval ($P<0.05$). These were the only tissues analyzed that

showed a net loss of previously existing protein mass. Two days after infection, the protein mass of carcass (-14%), gastrocnemius muscle (-21%), tibialis anterior muscle (-23%), gastrointestinal tract (-15%), and skin (-16%) were lower ($P<0.001$) in infected compared to healthy rats (Table II-6). The sum of these differences represents an 1.77 g (-13%) difference in total body protein mass between the two treatment groups.

Infection substantially modified the proportions of total body protein represented by each tissue. Two days after infection, liver comprised a larger proportion (7.7% vs. 6.5%) ($P<0.05$), gastrocnemius and tibialis anterior muscle a smaller proportion (0.60% vs. 0.55%) ($P<0.05$) and (0.19% vs. 0.17%) ($P<0.05$), respectively, whereas skin and gastrointestinal tract did not change their proportions to any extent ($P>0.05$).

Nitrogen balance two days after infection, was compared to food intake one day after infection. Infected animals that ate the least had the lowest nitrogen balances ($r^2=.94$; $P<0.001$).

Catch-up Phase

Following the catabolic phase of infection, rats went through a period of accelerated growth, starting at approximately day 4 and apparently complete by day 18. In order to simplify the presentation of results, only detailed results for Expt 1 are shown; a summary of results of the catch-up phase of infection (day 4 to day 18) is presented for both Expt 1 and Expt 2 (Table II-7).

Infected rats began to increase in weight by day 2 (Figure II-1) (Appendix 1A & 1B) and gained weight at a faster rate than control

rats from day 1 through day 14. From regression analysis of body weight against day, control and infected rats gained 5.0 ± 0.13 and 6.1 ± 0.18 g/day ($P<0.001$). It can be seen from Figure II-1, that the growth curves intersected nine days after infection.

From day 4 to day 18, there were no differences in daily food intake between the two treatment groups (Table II-2 & II-7) (Appendix 1G). No compensation of food intake was made during the catch-up period; infected rats did not eat more food than control rats. Food intake was also not different ($P>0.05$) when expressed on a body weight basis (data not shown). The rate of urinary nitrogen excretion was not different between the two treatment groups between day 4 and day 18 ($P>0.05$) (Table II-7) (Appendix 1I). It was also not different ($P>0.05$) when expressed on a body weight basis (data not shown). Rats from the infected group excreted less faecal nitrogen at all time points between day 4 and 18 (Table II-5) (Appendix 1H); overall, this difference was statistically significant (-22%; $P<0.01$) (Table II-7).

The daily nitrogen balances of rats from the infected group tended to be higher than those from the control group at all time points between day 4 to day 18 (Table II-3) (Appendix 1D). Overall, between day 4 and day 18, nitrogen balance was 10% higher in rats from the infected group ($P<0.06$) (Table II-7). In terms of cumulative nitrogen balance, rats from the infected group caught up to controls by day 18 (Expt 1) (Figure II-2) (Apendix 1E). Rats in Expt 2 caught up by day 23.

At all time points between day 4 and day 18, the apparent nitrogen digestibility was higher in rats from the infected group

(Table II-4) (Appendix 1G). Overall, during this period, the digestibility was higher (+9%; $P<0.01$) (Table II-7). From day 4 to day 18, there were no significant differences in apparent efficiency of nitrogen utilization between the two groups (Table II-7).

Determination of protein mass confirmed that catch-up had been achieved. The whole body protein mass of rats that were previously infected was not different ($P>0.05$) from that of control rats at day 18 ($32.8 \pm 1.9$ vs. $30.5 \pm 1.3$ g) (Expt 1). There were no differences ($P>0.05$) in the protein masses of gastrocnemius muscle, liver, gastrointestinal tract, and skin between the two treatment groups on day 23 (Expt 2) (Table II-6). There were also no differences ($P>0.05$) in the RNA and DNA masses and the ratio of RNA/DNA of gastrocnemius muscle between the two treatment groups on day 23 (Appendix 1J).

Direct measures of carcass protein and nitrogen balance measurements agreed well. For example, based upon direct carcass analysis, rats gained approximately 20 g of whole body protein between day 0 and day 18; based upon nitrogen balance, rats gained about 21 g protein.

Post catch-up Phase

There were no differences ($P>0.05$) in body weight, food intake, faecal nitrogen excretion, urinary nitrogen excretion, daily nitrogen balance, cumulative nitrogen balance, and apparent efficiency of nitrogen utilization between the two groups between day 18 and day 44 (Appendix 1A to 1I). There were no differences ($P>0.05$) in whole body protein masses between control and previously infected rats on day 44 ($43.1 \pm 1.8$ g vs. $42.3 \pm 2.1$ g).

# DISCUSSION

## Catabolic Phase

In the present study, *E. coli* infection caused a marked depression in body weight, food intake, daily nitrogen balance, and carcass and tissue protein masses. Depressed nitrogen balance two days after infection was associated with decreased food intake. This does not necessarily imply that intake alone was sufficient to account for the lower nitrogen balance. Although urinary nitrogen per rat was not different between the two groups per se, urinary nitrogen was maintained in infected rats even though nitrogen supply was severely depressed. This resulted in infected rats not being able to retain nitrogen as efficiently as controls and a reduction in protein accretion. Faecal nitrogen excretion decreased with decreased food intake. There was also a small reduction in digestibility in Expt 2, which would further decrease the amount of nitrogen available to the body. This description of depressed nitrogen balance in the whole body, was further elaborated at the level of specific tissues.

A most interesting finding was that skin and gastrointestinal tract, in addition to skeletal muscle, were greatly affected by infection. Skeletal muscle and gastrointestinal tract lost previously existing protein, while protein accumulation in skin was halted acutely after infection. These changes may have a substantial effect on function of tissues such as the gastrointestinal tract, which is metabolically active and plays a central role in maintaining nutrient and immune status.

Of the decrease in whole body protein mass of infected rats (at day 2), 66% was from carcass, 27% from skin, and 6% from gastrointestinal tract. Although total skeletal muscle mass was not measured, the majority of the difference in carcass protein is probably attributable to this tissue, because the other organs included in the carcass (heart, lungs, kidney, spleen) were unlikely to have lost protein (Fern et al. 1985). Skeletal muscle and skin appear to be, quantitatively, the major tissues capable of reduced nitrogen balance during infection. Skeletal muscle is a primary site of protein mobilization in diverse catabolic states (Rennie 1985, Wannemacher et al. 1975). Although skin protein was not mobilized, net protein synthesis in this tissue was negligible during the early part of infection. Interestingly, similar effects were seen in our recent study of muscle and skin during negative nitrogen balance associated with lactation (Baracos et al. 1991). Skin protein metabolism, thus, appears to have considerable plasticity and to be coordinately regulated with whole-body nitrogen economy. The extent to which changes in skin protein mass affect the functional integrity of this organ remains unclear.

Catch-up Phase

Infected rats grew at a faster rate than age-matched controls, so by all the criteria measured, they were able to catch up. Catch-up in terms of nitrogen balance took longer than for body weight. When body weights were equivalent, the cumulative nitrogen balance was substantially lower in infected rats. The ability to catch up may not be universal. In the case of nutrient deprivation, catch-up is

dependent upon the type, severity, and duration of the insult; the same criteria may apply to infection. Following nutrient deprivation, the ability of skeletal muscle to recover is dependent upon its ability to achieve a normal complement of DNA (Glore and Layman 1987, Beermann 1983). Age and species may also affect the ability to catch up. For example, eight day old chicks infected with E. coli had not caught up, in terms of body weight, four weeks after infection (Tian and Baracos 1989a).

Comparisons between aspects of protein metabolism during catch-up growth following infection and nutrient deprivation, for which there is a substantial literature, are informative. Catch-up can occur by increasing the amount of nitrogen available to the body or by increasing the metabolic efficiency with which nitrogen is used. During catch-up growth, infected rats ate no more food on a total or per g body weight basis, than controls. By contrast, animals recovering from food deprivation increased the total or the relative amount of food eaten (Wilson and Osbourn 1960, Widdowson and McCance 1963). Rats that were infected did not catch up by decreasing urinary nitrogen, in contrast to animals recovering from nutrient deprivation, which decreased urinary nitrogen excretion (Whittemore et al. 1978). In addition, the apparent efficiency of utilization of nitrogen did not increase significantly during the catch-up period. Recovery from nutrient deprivation is associated with an increase in efficiency (Whittemore et al. 1978). During catch-up, the apparent digestibility of nitrogen was consistently higher in infected rats. Following nutrient deprivation, recovery has not been shown to be associated with an increase in apparent or true digestibility of

nitrogen (Wilson and Osbourn 1960, Whittemore et al. 1978). Apparently, some of the mechanisms by which catch-up occurs after infection are distinct from those following nutrient deprivation. These may also vary between infections of different types. Consequently, conditions for optimizing catch-up growth may differ depending on the clinical state.

Mechanisms of Catch-Up

In our study, a principal means of increased nitrogen balance was increased apparent nitrogen digestibility. The mechanism by which this variable is increased during recovery from infection is unknown, but may occur by increasing transit time, increasing the absorptive surface, or by increasing the permeability of the small intestine. This may also occur by decreasing endogenous nitrogen secretion, which would increase apparent digestibility but not increase true digestibility of dietary nitrogen. These possibilities remain to be tested.

The marked changes in tissue protein mass which we observed must result from alterations in protein synthesis and/or degradation. There is very little information regarding protein turnover in different tissues during catch-up. For example, skeletal muscle protein synthesis has been shown to increase during recovery from acute starvation (Garlick et al. 1983); increased insulin responsiveness has been shown to be one of the mechanisms responsible for this anabolic effect (Garlick et al. 1983). Measurements of protein synthesis and degradation, and their responses to various anabolic and catabolic signals, in individual tissues would be

required to determine their relative contributions to protein repletion during catch-up.

Methodological Considerations

The objective of this research was to study catch-up growth following infection. This required a model that would produce a sufficiently marked and reproducible effect, and one in which catch-up growth could be detected. There are a variety of experimental models of infection (Wichterman et al. 1980), including chronic and acute infections, and the use of endotoxin. Many of these approaches are unsuitable. Endotoxin while reproducible, is an acute treatment that does not adequately simulate many metabolic changes produced by infection. Many infections are either variable, unreproducible, or are lethal (Wichterman et al. 1980). We have used a single injection of E. coli to study growth and protein metabolism in 250 g rats and chicks (Tian and Baracos 1989a,b). This infection proved to be useful in weanling rats. Complete catch-up occurred over a reasonable and convenient time period (~3 weeks). Death loss varied, despite using a single dose of bacteria, obtained from a constant source, and grown under identical conditions. Lowering the dose in Expt 3 proved to be as effective in generating a catabolic effect, without any mortality. The tight correlation between food intake and nitrogen balance immediately after infection indicated that food intake could be used as a simple indicator of nitrogen balance and thus of the catabolic effect. This information could be used in future experiments to select animals showing a uniform catabolic response.

We studied body weight, nitrogen balance, and carcass and tissue protein mass. Body weight appeared to be a poor indicator of catch-up. Nitrogen balance measures have the advantage that they provide information about whole body nitrogen economy over a continuous period, which can then be verified by direct measurements of carcass and tissue protein. Measures of individual tissues indicate that changes in whole body nitrogen may mask organ effects of an anabolic or catabolic nature. Examination of individual tissues, therefore, is crucial.

## Conclusion

*E. coli* peritonitis in weanling rats proved to be a useful model for studying catch-up growth after infection. Catch-up occurred by increasing nitrogen digestibility, rather than by increasing the apparent efficiency of nitrogen use in the whole body. Further experiments are required to clarify the mechanisms by which catch-up growth occurs. Our ongoing work involves studies of protein turnover in individual tissues during catch-up from infection.

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Table II-1 Diet for weanling rats[1]

| Ingredient | g/kg diet |
|---|---|
| ground corn | 584 |
| soybean meal | 320 |
| DL-methionine[2] | 2 |
| corn oil[3] | 45 |
| AIN-76 vitamin mix[4] | 10 |
| AIN-76 mineral mix[5] | 35 |
| choline chloride[6] | 1 |
| $CaCO_3$[7] | 3 |
| Total | 1000 |

crude protein 20% gross energy 17 MJ/g

1 As fed basis.

2 DL-Methionine, Philipp Brothers (Canada) Ltd, 1440 Ste. Catherines St., Montreal, Quebec, Canada.

3 Corn Oil, Crown-Best Foods, Etobicoke, Ontario, Canada.

4 AIN-76 vitamin mix, Teklad. As per kg of mixture: thiamine hydrochloride, 600.0 mg; riboflavin, 600.0 mg; pyridoxine hydrochloride, 700.0 mg; nicotinic acid, 3.0 g; D-calcium pantothenate, 1.6 g; folic acid, 200.0 mg; D-biotin, 20.0 mg; cyanocobalamin, 1.0 mg; retinyl palmitate pre-mix (250,000IU/g), 1.6 g; DL-alpha-tocopherol acetate pre-mix (250 IU/g), 20 g; cholecalcipherol (400,000 IU/g), 250.0 mg; menaquinone, 5.0 mg; sucrose, finely powdered, 972.9 g.

5 AIN-76 mineral mix, Teklad. As per kg of mixture: calcium phosphate dibasic, 500.00 g; sodium chloride, 74.00 g; potassium citrate monohydrate, 220.00 g; potassium sulphate, 52.00 g; magnesium oxide, 24.00 g; manganous carbonate (43-48% Mn), 3.50 g; ferric citrate (16-17% Fe), 6.00 g; zinc carbonate (70% $ZnCO_3$), 1.60 g; cupric carbonate (53-55% Cu), 0.30 g; potassium iodate, 0.01g; sodium selenite, 0.01 g; chromium potassium sulphate, 0.55 g; sucrose, finely powdered, 118.00 g.

6 Choline chloride (60%), Chinook Group, Inc., North Branch, Minnesota, U.S.A.

7 Calcium carbonate, Limeco Products Ltd., Rocky Mountain House, Alberta, Canada.

Table II-2 Daily food intake[1]

| Time Period | Control (n≥6) | Infected (n≥7) | % Change | P |
|---|---|---|---|---|
| (days) | (g/rat/day) | | | |
| 0 to 1 | 13.5 ± 0.5 | 4.7 ± 0.6 | -65.2 | <0.001 |
| 1 to 2 | 12.2 ± 0.3 | 8.1 ± 0.3 | -33.6 | <0.001 |
| 2 to 4 | 12.9 ± 0.3 | 11.6 ± 0.3 | -10.1 | <0.01 |
| 4 to 6 | 14.7 ± 0.4 | 14.2 ± 0.3 | -3.4 | NS[2] |
| 6 to 8 | 14.5 ± 0.4 | 14.9 ± 0.5 | +2.8 | NS |
| 8 to 10 | 15.6 ± 0.8 | 14.7 ± 0.5 | -5.8 | NS |
| 10 to 14 | 14.9 ± 0.5 | 14.9 ± 0.5 | 0 | NS |
| 14 to 18 | 15.4 ± 0.5 | 15.2 ± 0.6 | -1.3 | NS |
| 18 to 25 | 14.8 ± 0.7 | 14.9 ± 0.5 | +0.7 | NS |
| 25 to 32 | 15.9 ± 0.5 | 16.3 ± 0.9 | +2.5 | NS |
| 32 to 39 | 15.6 ± 0.4 | 15.1 ± 0.6 | -3.2 | NS |
| 39 to 44 | 16.3 ± 0.7 | 16.0 ± 0.7 | -1.8 | NS |

Note. Differences between means were assessed by a Student's t-test.

[1]On day 0, animals were given a single i.p. injection of *E. coli* in sterile saline ($\sim 10^9$ CFU/kg body weight), or vehicle alone.

[2]NS = not significant.

Table II-3 Daily nitrogen balance

| Time Period | Control (n≥6) | Infected (n≥7) | % Change | P |
|---|---|---|---|---|
| | | (mg/rat/day) | | |
| 0 to 2 | 251 ± 12 | 90 ± 12 | -64.1 | <0.001 |
| 2 to 4 | 237 ± 8 | 224 ± 11 | -5.5 | NS |
| 4 to 6 | 227 ± 10 | 248 ± 6 | +9.3 | NS |
| 6 to 8 | 203 ± 12 | 231 ± 17 | +14.0 | NS |
| 8 to 10 | 204 ± 14 | 221 ± 18 | +8.3 | NS |
| 10 to 14 | 158 ± 9 | 183 ± 10 | +15.8 | NS |
| 14 to 18 | 130 ± 7 | 141 ± 8 | +8.5 | NS |
| 18 to 25 | 132 ± 9 | 141 ± 9 | +6.8 | NS |
| 25 to 32 | 181 ± 13 | 165 ± 18 | -8.8 | NS |
| 32 to 39 | 147 ± 7 | 129 ± 12 | -12.2 | NS |
| 39 to 44 | 160 ± 18 | 151 ± 28 | -5.6 | NS |

Note. Differences between means were assessed by a Student's t-test.

Table II-4 Apparent nitrogen digestibility[1]

| Time Period | Control (n≥6) | Infected (n≥7) | % Change | P |
|---|---|---|---|---|
| (days) | | (%) | | |
| 0 to 2 | 79.2 ± 1.5 | 78.1 ± 1.2 | -1.4 | NS |
| 2 to 4 | 78.5 ± 2.3 | 84.2 ± 0.8 | +7.3 | <0.05 |
| 4 to 6 | 75.2 ± 2.4 | 80.5 ± 1.0 | +7.0 | <0.05 |
| 6 to 8 | 75.2 ± 3.1 | 81.7 ± 0.9 | +8.6 | <0.05 |
| 8 to 10 | 73.2 ± 4.1 | 83.3 ± 1.2 | +13.8 | <0.05 |
| 10 to 14 | 72.1 ± 3.1 | 76.4 ± 1.7 | +6.0 | NS |
| 14 to 18 | 65.0 ± 4.1 | 74.3 ± 2.2 | +14.3 | NS |
| 18 to 25 | 60.6 ± 3.4 | 74.1 ± 2.4 | +22.3 | <0.05 |
| 25 to 32 | 75.7 ± 1.9 | 79.4 ± 1.6 | +4.9 | NS |
| 32 to 39 | 80.7 ± 0.7 | 80.7 ± 1.5 | 0 | NS |
| 39 to 44 | 80.2 ± 0.9 | 81.4 ± 0.5 | +1.5 | NS |

Note. Differences between means were assessed by Student's t-tests.

[1]Apparent nitrogen digestibility (%) = (nitrogen intake - faecal nitrogen)/ nitrogen intake x 100.

Table II-5 Daily faecal nitrogen excretion

| Time Period | Control (n≥6) | Infected (n≥7) | % Change | P |
|---|---|---|---|---|
| (days) | (mg/rat/day) | | | |
| 0 to 2 | 85 ± 6 | 44 ± 2 | -48.2 | <0.001 |
| 2 to 4 | 88 ± 9 | 58 ± 3 | -34.0 | <0.01 |
| 4 to 6 | 116 ± 10 | 89 ± 5 | -23.2 | <0.05 |
| 6 to 8 | 115 ± 15 | 88 ± 7 | -23.5 | NS |
| 8 to 10 | 140 ± 28 | 79 ± 6 | -43.6 | <0.05 |
| 10 to 14 | 132 ± 14 | 114 ± 10 | -13.6 | NS |
| 14 to 18 | 172 ± 20 | 128 ± 15 | -25.6 | NS |
| 18 to 25 | 187 ± 20[1] | 126 ± 16 | -61.0 | NS |
| 25 to 32 | 124 ± 12 | 110 ± 14 | -11.3 | NS |
| 32 to 39 | 97 ± 5 | 95 ± 10 | -0.4 | NS |
| 39 to 44 | 104 ± 9 | 95 ± 6 | -9.4 | NS |

Note. Differences between means were assessed by Student's t-tests.

[1] n = 5.

Table II-6 Carcass and tissue protein masses

| | Day 0[1] Control (n=6) | Day 2[1] Control (n=6) | Day 2[1] Infected (n=9) | Day 23[2] Control (n=15) | Day 23[2] Infected (n=5) |
|---|---|---|---|---|---|
| | | | (g protein/tissue) | | |
| Carcass[3] | 7.23 ±0.15 | 8.36[a*] ±0.18 | 7.19[b] ±.12 | 20.5[a] ±0.5 | 21.6[a] ±0.5 |
| Liver | 0.77 ±0.03 | .85[a*] ±0.03 | .88[a*] ±0.02 | 1.79[a] ±0.05 | 1.84[a] ±0.10 |
| Skin | 2.64 ±0.09 | 3.03[a*] ±0.08 | 2.55[b] ±0.06 | 5.77[a] ±0.30 | 5.77[a] ±0.16 |
| GIT[4] | 0.73 ±0.02 | 0.76[a] ±0.01 | 0.65[b*] ±0.02 | 1.31[a] ±0.02 | 1.27[a] ±0.02 |
| | | | (mg protein/tissue) | | |
| Gastrocnemius | 68.1 ±1.6 | 78.7[a*] ±1.2 | 62.3[b*] ±1.2 | 245[a] ±5 | 250[a] ±8 |
| Tibalis anterior | 21.5 ±0.5 | 24.5[a*] ±0.4 | 18.9[b*] ±0.6 | - | - |
| | | | (g protein/rat) | | |
| Total | 11.5 ±0.2 | 13.2[a*] ±0.3 | 11.4[b] ±0.1 | 29.9[a] ±0.6 | 31.0[a] ±0.6 |

Note. Differences between means were determined by Student's t-tests.
[1]Expt 3.
[2]Expt 2.
[3]Carcass minus gastrointestinal tract, liver, gastrocnemius muscles, hair, and skin.
[4]Rinsed gastrointestinal tract, distal oesophagus to anus.
[ab]Values with different superscripts within a tissue and within a day are significantly different ($P<0.05$).
* Significantly different from day 0.

Table II-7 Summary of catch-up phase - day 4 to 18

| | Expt 1 | | Expt 2 | | |
|---|---|---|---|---|---|
| | Control (n=11) | Infected (n=11) | Control (n=15) | Infected (n=5) | P |
| | | | (g/rat/day) | | |
| Daily Food Intake | 15.1 ±0.42 | 14.9 ±0.42 | 15.4 ±0.25 | 14.9 ±0.43 | NS |
| | | | (mg/rat/day) | | |
| Daily Faecal N | 140 ±10 | 106 ±8 | 141 ±5 | 113 ±7 | <0.01 |
| Daily Urinary N | 169 ±19 | 178 ±4 | 155[1] ±8 | 150 ±11 | NS |
| Daily N Balance | 175 ±7 | 193 ±9 | 197 ±6 | 215 ±14 | <0.06[2] |
| | | | (%) | | |
| Apparent N Digestibility | 70.7 ±2.3 | 78.0 ±1.2 | 71.4 ±1.1 | 76.4 ±1.2 | <0.01 |
| Efficiency of N Utilization | 52.6 ±3.4 | 51.8 ±1.5 | 56.1 ±1.5 | 58.9 ±3.0 | NS |

Note. Differences between means were determined by ANOVA.
[1] n = 10.
[2] By regression analysis

Figure II-1 Body weight of rats expressed as a percentage of day 0 body weight. Day 0 body weight was 87.5 ± 1.3 g. Weanling rats were infected on Day 0. Points are means ± SEM; control (--o--) (n=11); infected (—●—) (n=11). An 'a' denotes significant differences between treatment means ($P<0.05$).

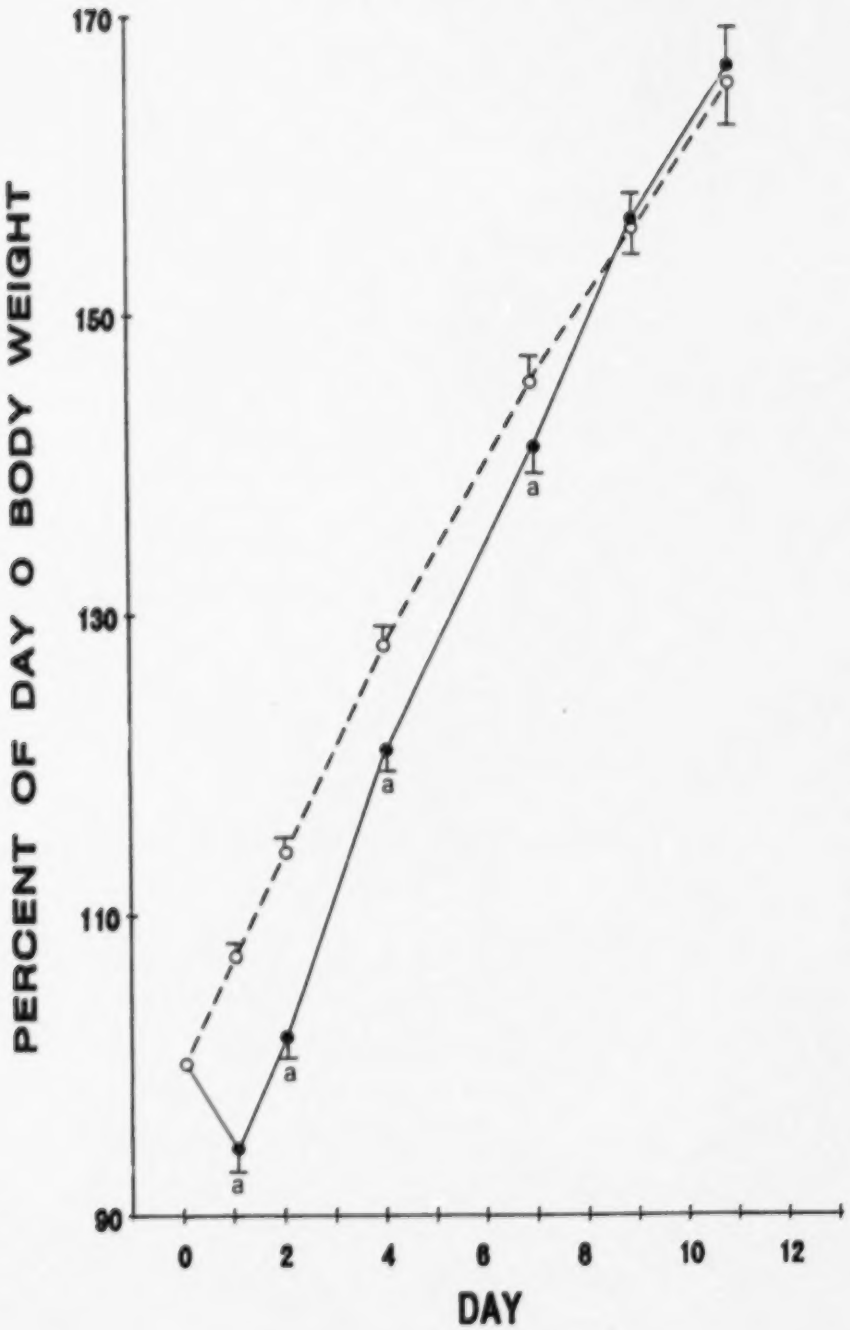
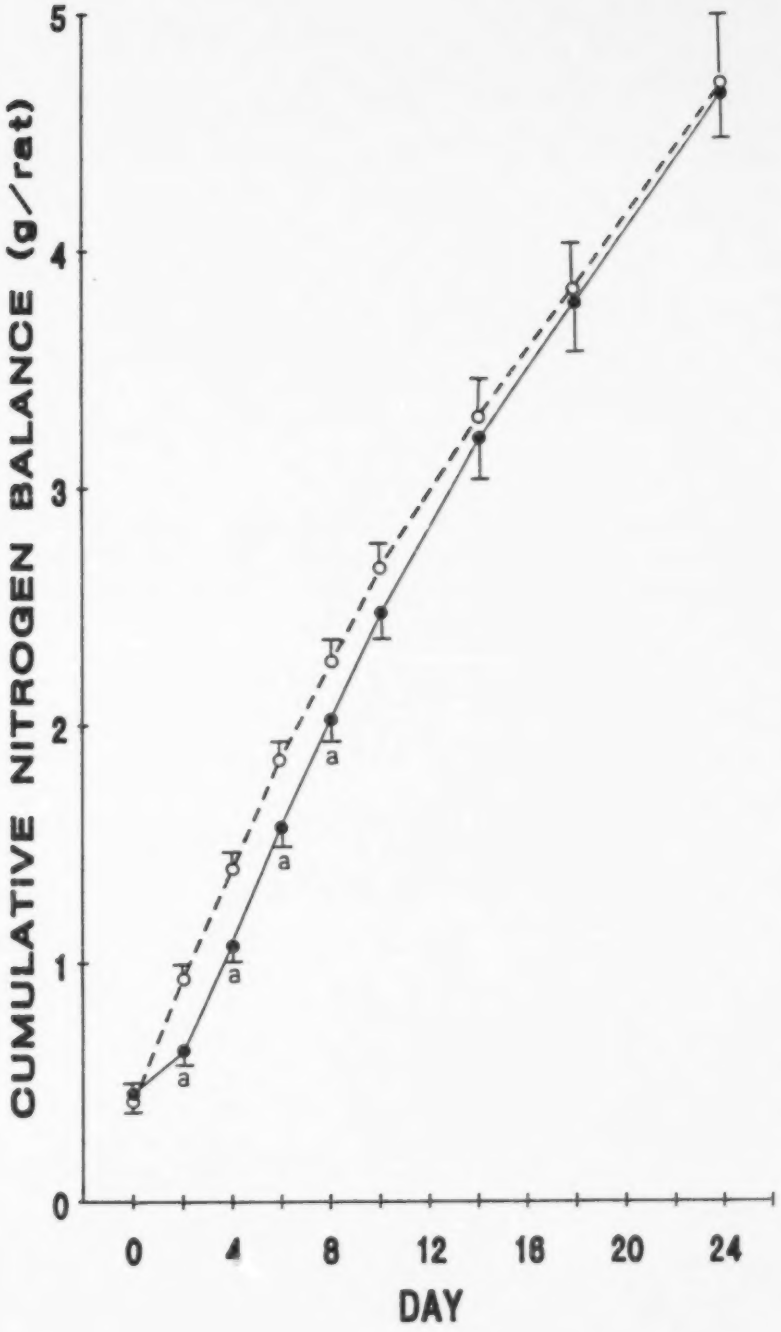

PERCENT OF DAY O BODY WEIGHT
170
150
130
110
90
0
2
4
6
8
10
12
DAY
a
a
a
a

Figure II-2 Cumulative nitrogen balance of rats. Rats were infected on day 0. Points are means ± SEM; control (--o--) ($n \geq 6$); infected (—●—) ($n \geq 7$). An 'a' denotes significant differences between treatment means ($P<0.05$).


CUMULATIVE NITROGEN BALANCE (g/rat)
5
4
3
2
1
0
a
a
a
a
0
4
8
12
16
20
24
DAY

# III. TISSUE PROTEIN TURNOVER DURING CATCH-UP GROWTH FOLLOWING ESCHERICHIA COLI INFECTION IN WEANLING RATS

## A. INTRODUCTION

Poor growth and muscle protein wasting are a hallmark of infection (Beisel 1984). Total recovery from infection involves the animal's return to its original growth curve and achievement of normal body protein mass and proportion. This involves growth at a rate faster than healthy animals of the same age, and is defined as catch-up growth (Tanner 1986). Catch-up growth following infection has been the subject of little research.

Recently, we have developed a simple and practical model to study the effects of infection on growth and protein metabolism (Samuels and Baracos 1992). Our model uses E. coli peritonitis in weanling rats, which has a marked effect on whole body and tissue protein status. In our studies, complete catch-up growth, as measured by nitrogen balance and tissue protein mass, occurred within 3 weeks after infection (Samuels and Baracos 1992). On a whole body basis, this was achieved by augmenting nitrogen balance by increased apparent nitrogen digestibility, whilst nitrogen intake and urinary nitrogen excretion remained unchanged. This phenomenon was shown to be most pronounced between days 4 and 14 post-infection (Samuels and Baracos 1992). Nitrogen balance measurements, however, do not address how nitrogen metabolism of individual tissues respond. In our previous studies, tissues were affected differently (Samuels and

[1] A version of this chapter has been submitted for publication. S. E. Samuels and V. E. Baracos. 1992. Am. J. Physiol.

Baracos 1992). For example, two days after infection, skeletal muscle had -22% less protein mass and comprised a smaller proportion of total body protein than in healthy controls of the same age. Liver protein mass was not depleted, although its proportion of total body protein increased. We also discovered that the protein mass of skin and gastrointestinal tract was substantially lower (-16% and -15%, respectively) in infected rats compared to healthy controls. Thus different tissues must undergo different amounts of catch-up growth, and so the events leading to tissue protein repletion must be assessed on an individual tissue basis.

Enhanced tissue protein accretion must result from changes in tissue protein synthesis and/or degradation. Currently, there is no information regarding tissue protein turnover during catch-up growth. Therefore, the objectives of these experiments were to measure protein turnover in skeletal muscle (gastrocnemius and tibialis anterior), skin, small intestine, and liver in rapidly growing rats, during catch-up growth following infection. These tissues were selected in anticipation that 80% of whole body protein turnover would be accounted for (Waterlow 1984).

## B. METHODS

### Experimental Animals and Treatments

Studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Weanling female Sprague-Dawley rats, 22 to 24 days old were obtained from Biosciences Animal Services, University of Alberta. Rats were housed in individual wire mesh cages in rooms that were maintained at 26$^{o}$C on a 12 hour

light/dark cycle (lights on 0800 hours). Animals were given free access to food and water. The 20 % protein diet was formulated and prepared in our laboratory (Samuels and Baracos 1992). Animals were given six days to adjust to their new food and environment before treatments were imposed.

The effects of the strain of E. coli used were previously characterized in the weanling rat (Samuels and Baracos 1992), including: suitable dose, body weight, food intake, and impact on host nitrogen balance. A dose of $3.5 \times 10^9$ colony forming units/kg body weight had a marked effect on whole body and tissue nitrogen status without causing a substantial mortality. Rats were given the dose in a single intraperitoneal injection in 0.5 mL of sterile phosphate buffered saline, or vehicle alone.

Experimental Design

A total of 78 rats (32 control and 46 infected) were randomly allocated to treatments on the day of infection. Body weight and food intake were measured every one to two days. Protein turnover was measured in skeletal muscle (gastrocnemius and tibialis anterior), skin, liver, and small intestine. Gastrocnemius and tibialis anterior were selected because they are of mixed fibre type, which is representative of the majority of skeletal muscles in the body (Maltin et al. 1989). Pair-fed controls were not used because preliminary experiments showed that protein mass from pair-fed rats was not significantly different ($P>0.05$) from full-fed controls (Refer to Appendix 4). Protein turnover was measured on days 4, 6, 8, 11, and 14 after infection; these time points coincided with the

interval when nitrogen balance in infected rats was most augmented (Samuels and Baracos 1992). Three to twelve control and infected rats were used at each time point. The experiment was carried out in two replicates; data from both replicates were pooled.

A further experiment to measure protein degradation in skeletal muscle *in vitro* was performed. Twenty weanling female Sprague-Dawley rats were injected with *E. coli*, or saline alone, as described above. Four days after infection, total and fractional rates of protein degradation were measured in extensor digitorum longus (EDL) and epitrochlearis muscles (Epi); gastrocnemius and tibilis anterior are too large for *in vitro* studies . This corresponded to the time point in *in vivo* studies when the difference in protein degradation between control and infected rats was maximal.

Measurement of Protein Turnover In Vivo

Protein synthesis was determined using the flooding-dose method of Garlick et al. (1980). Refer to Appendix 5 for a detailed protocol. Between 1100 hours and 1300 hours on the day of study, rats were given an intraperitoneal injection of 150 μmoles phenylalanine (Phe) containing 50 μCi L-(2,6-$^{3}$H) Phe (Amersham, U.S.A.) in 1.25 mL of sterile phosphate buffered saline per 100 g body weight. After precisely 15 minutes, rats were killed by cervical dislocation. Their visceral cavities were quickly cut open and immediately immersed in ice-cold water. One tibialis anterior, one gastrocnemius (including plantaris), the liver, and a 1 to 2 g sample of jejenum (rinsed in ice-cold saline) and skin were rapidly removed, blotted, and frozen in liquid nitrogen. All tissues were

removed and frozen in less than 3 minutes. In addition to the above samples, the contralateral muscles (for Phe and RNA determination) were dissected intact, blotted, and frozen in liquid nitrogen; the rest of the skin was shaved, removed, and weighed for subsequent protein determination. Samples were stored at -50°C until analysed.

The specific radioactivity of free ($S_A$) and protein bound ($S_B$) Phe was measured in each tissue. The fractional rate of protein synthesis ($k_{syn}$) as %/day was calculated as: $k_{syn} = (S_B \times 100)/S_A \times t$, where t is the time between injection and slaughter (in days). $k_{growth}$ was calculated by a two step process. First, a linear regression ($P<0.05$) of tissue Phe or protein mass against body mass was performed for all animals for each treatment group. Then a regression of body mass against time was performed for each animal over the preceeding three to four days prior to slaughter; these regressions, in this and previous experiments (Samuels and Baracos 1992), were linear ($P<0.05$). By multiplying these slopes, the Phe or protein mass gained per day for each animal was calculated (Attaix and Arnal 1987). Fractional degradation rate ($k_{deg}$) was calculated by subtracting fractional growth rate ($k_{growth}$) from $k_{syn}$.

The absolute rate of protein synthesis (SYN), degradation (DEG), and growth (GROWTH) were calculated by multiplying the fractional rates by the Phe mass (skeletal muscles and liver) or the protein mass (skin) of that tissue. The efficiency of protein synthesis ($k_{RNA}$) was determined by dividing SYN by the RNA mass of that tissue. The capacity for protein synthesis ($C_s$) was calculated by dividing the RNA mass by the Phe mass of that tissue.

Measurement of Skeletal Muscle Protein Degradation In Vitro

Rats were killed by cervical dislocation and muscles were rapidly dissected. EDL were mounted on stainless steel supports at their resting length *in situ*. Total protein degradation (DEG) was measured as the release of Phe from muscles in the presence of 0.5 mM cycloheximide (Tischler et al. 1982). Muscles were pre-incubated in 3 mL of Krebs-Ringer bicarbonate medium composed of (in mM) 119 NaCl, 4.82 KCl, 1.25 $MgSO_4$, 1.0 $CaCl_2$, 1.24 $NaH_2PO_4$, 25 $NaHCO_3$, 2.0 N-2-hydroxoethylpiperazine-N'-2-ethanesulphonic acid (HEPES)/ NaOH, pH 7.4, 5.0 glucose, 0.85 leucine, 0.5 isoleucine, and 1.0 valine, plus 0.5 U insulin/mL. The contralateral EDL muscles were incubated in the same media except no insulin was added. The media were continually bubbled with 95%$O_2$-5%$CO_2$ and maintained at 35°C. After 30 minutes, muscles were transferred to fresh media of the same composition and incubated for 3 hours. Phe in the incubation medium, intracellular fraction, and the protein bound fraction were measured. Protein degradation was expressed as μg Phe released x muscle$^{-1}$ x 3 hour$^{-1}$ and as μg Phe released x 100 μg protein-bound Phe$^{-1}$ x d$^{-1}$ (%/day). See Appendix 6 for details.

Analyses

For the analysis of *in vivo* protein synthesis, tissue samples were prepared in a manner similar to that reported by Garlick et al. (1980). Refer to Appendix 5 for a detailed protocol. Half a gram of shaved skin, while semi-frozen, was minced with a scalpel and pulverized in a mortar and pestle with liquid nitrogen. The entire liver, small intestinal sample, and muscles were individually

pulverized in a mortar and pestle with liquid nitrogen. Entire muscles and about 0.5 g samples of other tissues were homogenized on ice with approximately 2 mL ice-cold 2% $HClO_4$ using a motorized ground glass tissue homogenizer, and then centrifuged for 15 minutes at 2000 g and 0°C.

Free and protein bound Phe specific radioactivity were measured after transformation to ß-phenylethylamine as described in detail by Baracos et al. (1991). Phe mass (skeletal muscles and liver) was measured by HPLC (Jones and Gilligan 1983). Protein mass (skin) was measured by the Kjeldahl method (AOAC 1984) after it was autoclaved and homogenized. RNA was measured by the method of Munro and Fleck (1969) (Appendix 3). All analyses were performed in duplicate.

Data Analysis

The effects of treatment, day, and treatmentxday were tested using ANOVA (SAS 1988). When overall significance of the model and main effects or interaction reached the 0.05 level, differences between means were determined using t-tests. Variability of tabulated data is expressed as root error mean square (rEMS); other values are means ± SEM.

## C. RESULTS

Body Weight and Food Intake

Healthy control rats showed rapid growth throughout the experimental period. One day after infection, body weight was 11.5% lower and food intake was 63% lower in infected compared to control rats (data not shown). Body weight began to increase after day 2 and

food intake returned to normal by day 4 (data not shown). These effects were similar to those previously reported (Samuels and Baracos 1992).

Skeletal Muscle Protein Turnover

Protein turnover data for tibialis anterior and gastrocnemius are shown in Tables III-1 and III-2. In tibialis anterior from healthy rats, about 700 μg of protein-bound Phe accumulated between days 4 and 14, which represented an increase of 50%. Daily Phe accumulation decreased ($P<0.05$) from about 90 to 60 μg Phe/day over 10 days. The absolute rate of protein synthesis was constant at ~200 μg Phe/day, and since Phe mass increased over time, $k_{syn}$ decreased ($P<0.05$) from 14.3 to 9.0 %/day. There was a small increase ($P<0.05$) in RNA mass from about 29 to 34 μg RNA-P, but no change ($P>0.05$) in $k_{RNA}$ (~6 μg Phe/μg RNA-P/day) over the 10 days of study. $C_s$ decreased ($P<0.05$) from ~21 to 15 μg RNA-P/mg Phe. The absolute rate of protein degradation increased ($P>0.05$) from 114 to 141 mg Phe/day over 10 days. Because both Phe mass and protein degradation increased over time, there was no decrease in $k_{deg}$. The results in gastrocnemius from healthy rats were similar except the $k_{RNA}$ decreased ($P<0.05$) over time.

Early in catch-up growth (day 4), infected rats had lower ($P<0.05$) Phe masses in tibialis anterior (1.15 ± 0.05 vs. 1.41 ± 0.05 mg) and gastrocnemius (3.17 ± 0.13 vs. 3.93 ± 0.11 mg) compared to controls. Tibialis anterior and gastrocnemius Phe masses in infected rats also comprised a smaller proportion of body weight (1.1 vs. 1.3; $P<0.05$ and 3.0 vs. 3.7 mg Phe/100 g body weight; $P<0.05$).

During catch-up growth, skeletal muscle fractional and absolute rates of growth were higher ($P<0.001$) in infected rats. On day 4, the absolute rate of growth was 61% higher ($P<0.05$) in tibialis anterior and 40% higher in gastrocnemius ($P<0.05$). $k_{growth}$ was 92% higher ($P<0.05$) in tibialis anterior and 80% higher ($P<0.05$) in gastrocnemius of previously infected animals. These differences were evident subsequent to day 4, but decreased in magnitude and significance until day 14 when there were no treatment differences. There were no differences ($P>0.05$) in the absolute rate of protein synthesis between infected and control rats at any time point in either muscle. However, $k_{syn}$ in infected rats was 17 to 25% higher ($P<0.001$) between days 4 and 11 in both muscles. There were no treatment differences ($P>0.05$) by day 14. There were no treatment differences ($P>0.05$) in the RNA masses of either muscle at any time point. Because there were no differences in the absolute rate of protein synthesis and RNA mass, there were also no differences in $k_{RNA}$. Because of a lower Phe mass in infected rats, $C_s$ in this group was higher ($P<0.05$).

The absolute rate of protein degradation in infected rats was 50% lower in tibialis anterior and 46% lower in gastrocnemius on day 4 ($P<0.05$). There were no other treatment differences between days 6 to 14. $k_{deg}$ was 41% lower ($P<0.05$) in tibialis anterior and 35% lower in gastrocnemius on day 4. Protein degradation data obtained in vitro are shown in Table III-3. On day 4, the protein-bound Phe mass of EDL and Epi in infected rats was lower ($P<0.001$) than in control rats (EDL: $1.74 \pm 0.05$ vs. $1.96 \pm 0.03$ mg; Epi: $0.69 \pm 0.03$ vs. $0.79 \pm 0.03$ mg). Total protein degradation was 16% and 11% lower

($P<0.05$) in EDL and Epi in infected compared with control rats. There were no differences in $k_{deg}$. There were no differences in intracellular free Phe between control and infected rats in EDL (data not shown); thus protein degradation values were based on Phe release only. The presence of insulin in the incubation media decreased ($P<0.05$) protein degradation 9.1% in control and 11.5% in infected EDL. There was no difference ($P>0.05$) in the insulin-stimulated decrease in protein degradation between control and infected rats.

Protein Turnover in Skin

In healthy control rats, skin protein mass increased ~0.22 g per day, resulting in a 40 % increase in protein mass from day 4 to 11 ($P<0.05$) (Table III-4). Absolute (~2 g/day) and fractional (~45%/day) rates of protein synthesis did not change over the study period ($P>0.05$). Absolute (~1.8 g/day) and fractional (~40%/day) rates of protein degradation also did not change over the study period ($P>0.05$).

Early in catch-up (day 4), infected rats had less ($P<0.05$) protein mass in skin compared to controls (3.04±0.18 vs. 3.97±0.18 g). Skin protein mass also comprised a smaller proportion of body weight in infected rats at this time (3.0 vs. 3.7 g protein/100 g body weight; $P<0.05$). During catch-up, skin growth, like that of muscle, was higher ($P<0.001$) in infected rats. On day 4, the absolute rate of growth was 95% higher ($P<0.05$) and $k_{growth}$ was 161% higher ($P<0.05$). These differences decreased thereafter. During catch-up, there were no differences in absolute rates of protein synthesis between infected and control rats. Unlike muscle, there were no treatment differences in $k_{syn}$ ($P>0.05$).

The absolute rate of protein degradation in infected rats was lower ($P<0.05$) in skin; it was 22% lower on day 4. This difference was smaller (-10 to -22%) and more prolonged than in skeletal muscle. There were no treatment differences in $k_{deg}$ ($P>0.05$).

Protein Turnover in Liver

In healthy control rats, about 17 mg of Phe accumulated in liver between days 4 and 14, which represented a 40 % increase. Daily Phe accumulation decreased ($P<0.05$) from 2.2 to 1.4 mg/day over 10 day of study (Table III-5). Absolute (~50 mg/day) and fractional (100 %/day) rates of protein synthesis did not change ($P>0.05$) between days 4 and 14.

On day 4, the Phe mass of liver in infected rats was not different from controls (43.0 ± 2.3 vs. 39.9 ± 2.9 mg). However, liver Phe mass comprised a larger proportion of body weight in infected rats (42 ± 2 vs. 38 ± 2 mg Phe/100g body weight; $P>0.05$). During the phase of catch-up growth where muscle and whole body growth were augmented, there were no treatment differences in the growth rate of liver. There were no differences in either the absolute ($P=0.14$) or fractional ($P=0.22$) rates of protein synthesis in liver, although they tended to be higher in infected rats. There were no differences ($P>0.05$) in RNA mass, $C_s$, and $k_{RNA}$.

Protein degradation was not calculated in liver because $k_{syn}$ comprises the synthesis of both exported and fixed proteins, whereas $k_{growth}$ does not include exported proteins.

Protein Synthesis in Small Intestine

In healthy control rats, $k_{syn}$ in small intestine decreased

($P<0.05$) from 101.3 to 78.9 %/day between days 4 and 14 (Table III-6). There were no differences ($P>0.05$) in $C_s$ (~115 μg RNA-P/ mg Phe) or $k_{RNA}$ (~7 μg Phe/μg RNA-P/day) over the 10 days of study.

On day 4, there was no difference in the protein content of the small intestine between control and infected rats (0.661 ± 0.018 vs. 0.682 ± 0.029 g; $P>0.05$). Small intestinal protein mass in infected rats comprised a larger proportion of body weight than controls (0.672 ± 0.019 vs. 0.612 ± 0.009 g protein/100 g body weight; $P<0.05$). Between days 4 and 14, $k_{syn}$ in small intestine (jejenum) was higher ($P<0.001$) in infected rats. There were no treatment differences ($P>0.05$) in the $C_s$ or $k_{RNA}$.

## D. DISCUSSION

### Growth and Protein Synthesis

Over the range of ages studied (32 to 46 days of age), rats grow rapidly and fractional rates of protein synthesis, degradation, and growth decrease. Most developmental work has focused on skeletal muscle, and the rates of protein turnover in skeletal muscles in this experiment fall within ranges reported by others (Millward et al. 1975, Garlick et al. 1980, Reeds et al. 1982, Preedy et al. 1983, El Haj et al. 1986, Jepson et al. 1986). Rates of protein turnover in other tissues agree with those in the literature (Garlick et al. 1980, Preedy et al. 1983, Reeds et al. 1982). In this and prior experiments (Baracos et al. 1991), we specifically measured Phe rather than protein mass, and thus our data cannot be directly compared to literature values. We chose to express tissue protein

mass as Phe, since this was the tracer used. Assuming muscle tissue protein contains 3.6% Phe (Odessey et al. 1974), our values for $k_{RNA}$ and $C_s$ are consistent with literature values (Millward et al. 1975, El Haj et al. 1986).

During catch-up growth, both the absolute and fractional rates of growth in muscle and skin were substantially elevated. The elevated growth rates of muscle, skin, and especially small intestine were most apparent very early in catch-up growth, and are astonishing in that only a few days earlier these tissues would have been in poor or negative nitrogen balance. An interesting finding was that small intestine had completely caught up by day 4. We previously showed that the gastrointestinal tract of infected rats had 15 % less protein mass than controls 2 days after infection (Samuels and Baracos 1992). Protein turnover would have to be measured prior to day 4 to determine the respective contributions of synthesis and degradation to protein repletion of this tissue. Because of the high rate of protein turnover in intestine, complete catch-up growth in a very short time would be possible with only small changes in protein turnover. Giving priority to catch-up growth of intestine makes sense because this would promote the functional capacity of intestine and thus digestion and absorption of dietary nitrogen to permit the subsequent catch-up growth of other tissues. By contrast, liver did not show net catabolism during infection (Samuels and Baracos 1992), and thus catch-up growth was not evident.

During catch-up growth, the absolute rate of protein synthesis was not modified in muscle or skin. Absolute rates of protein synthesis are dependent upon RNA mass and efficiency of protein

synthesis (Millward et al. 1975). Since neither RNA mass nor efficiency of protein synthesis in infected rats was different from controls, absolute rates of protein synthesis were maintained in infected rats. The protein mass in infected rats was lower; thus when the absolute rate of protein synthesis in muscle was expressed relative to Phe mass, the fractional rate of protein synthesis was significantly higher in these rats. By contrast, fractional rates of protein synthesis were not different in skin between control and infected rats. The apparent difference between the behavior of absolute rates of synthesis (no treatment effect) and fractional rates of protein synthesis (significant treatment effect) in muscle, therefore, results solely from alterations in tissue protein mass. Synthesis in most studies (Millward et al. 1975, Jepson et al. 1986, El Haj et al. 1986, Tian and Baracos 1989) is reported as fractional rates because determination of absolute rates require the additional measurement of protein or amino acid mass, or to normalize data in order to make comparisons among ages, species, etc. It is clear from the data reported here that erroneous conclusions could be drawn if only fractional rates had been considered.

Protein Degradation

Protein degradation can be measured directly in vitro; however, this method is only appropriate for certain thin skeletal muscles (Sugden and Fuller 1991). In vivo, protein degradation is measured indirectly, but such measures can be used in all tissues. Degradation in vivo is calculated as the difference between synthesis and growth (i.e., net protein gain). Growth is usually measured by

regression analysis of tissue protein mass of a large number of animals against time; this results in growth rates that are not calculated on an individual animal basis and statistical analyses cannot be performed (El Haj et al. 1986). Because we calculated growth on an individual animal basis, we were also able to calculate degradation on an individual animal basis, and thus apply statistics (Attaix and Arnal 1987).

Catch-up growth in muscle and skin was achieved by decreasing the absolute rate of protein degradation. Both in vivo and in vitro results obtained from muscles on day 4 supported this conclusion. In addition, in vivo (4.7 to 8.0 %/day) and in vitro (5.5 to 6.9 %/day) rates of protein degradation were similar to each other. Fractional rates of protein degradation were lower in muscle from infected compared to control rats in vivo but not in vitro. This difference possibly arose because the Phe mass of muscles from infected rats in the in vitro experiment was not decreased as much as those in the in vivo experiment; this would attenuate differences between treatments. Decreased fractional and absolute rates of protein degradation have also been observed during recovery from endotoxin administration. (Jepson et al. 1986, Tian and Baracos 1989). This mechanism appears to be unique, because accelerated rates of growth are usually associated with increased protein synthesis and degradation. For example, there is elevated absolute and fractional rates of muscle protein synthesis and degradation associated with stretch induced hypertrophy (Laurent et al. 1978) during the transient growth of the hemidiaphragm following unilateral phrenectomy (Turner and Garlick 1974), and the rapid growth of lean versus obese strains of rats (Reeds et al. 1982).

The decrease in muscle and skin proteolysis was most pronounced early in catch-up. Thus there is a rapid transition between increased protein breakdown, which is characteristic of the catabolic phase of infection (Kettlehut et al. 1988, Tian and Baracos 1989) and decreased degradation, which occurs in recovery. Data of Jepson et al. (1986) indicated that as rats shifted from a catabolic to an anabolic state following endotoxin administration, muscle protein degradation decreased six fold in 24 hours. Tissue proteolysis appears to have considerable plasticity in regulating protein balance in infection. In order to drive this rapid transition from a catabolic to anabolic state, there must be profound rapid changes in hormones or other factors, or in tissue sensitivities to these compounds, such as insulin or IGF-I. There may also be a rapid decline in catabolic factors such as glucocorticoids and macrophage products. Experiments monitoring the profile of these compounds through the catabolic and anabolic phases of infection would yield clues as to which factors are responsible for accelerated growth.

Protein degradation is precisely regulated by diet and hormones (Kettlehut et al. 1988). The biochemical pathways regulating both the activation and in this case the deactivation of protein degradation remain obscure. Muscle and skin contain multiple pathways for protein breakdown, including lysosomal, $Ca^{2+}$-dependent, and cytosolic ATP-dependent and independant proteolytic pathways (Kettlehut et al. 1988). Experiments measuring the relative contributions of the various proteolytic pathways would be required to resolve the participation of these systems.

Significance of Catch-up Growth Following Infection

From the present and previous work (Samuels and Baracos 1992), we have demonstrated that catch-up growth occurs on both a whole body and individual tissue basis following infection. Our experience has allowed us to define several unique aspects of this phenomenon. There was increased protein utilization, manifested at the level of dietary protein absorption, as well as in tissues. At the level of absorption, there was more nitrogen absorbed relative to the amount consumed (Samuels and Baracos 1992). In peripheral tissues, such as skin and muscle, which serve as reservoirs of amino acids during the catabolic phase of infection, there was increased net protein synthesis relative to total protein synthesis. These result in an overall increase in energetic and amino acid efficiency of protein deposition. This strategy makes sense, especially early in catch-up growth, because the animal may still be too weak to seek food and may not have fully recovered its appetite. In this and previous experiments, infected animals did not increase their food intake during catch-up growth (Samuels and Baracos 1992).

We also described the time course of changes that occurred during catch-up growth. The main adaptations occurred over discrete time periods and in distinct order. Increased whole body nitrogen balance was most pronounced from days 4 to 14 (Samuels and Baracos 1992). Protein lost from the small intestine was restored by day 4 and may be related in some way to the improved apparent nitrogen digestibility observed thereafter (Samuels and Baracos 1992). Catch-up growth in muscle and skin was pronounced early in catch-up growth but continued throughout the study. Thus, an accurate

characterization of catch-up growth following infection or any catabolic state involves studying a variety of time points.

While catch-up growth following infection occurred in our catabolic model, it may not always occur. This will likely depend on the type, severity, and duration of the stress as well as age, sex and species (Wilson and Osbourn 1960, Beisel 1984, Rennie 1985). For example, chicks infected with E. coli did not display enhanced tissue growth by 12 days after infection (Tian and Baracos 1989). In addition, if RNA mass were decreased, efficiency of protein synthesis would have to be increased to maintain protein synthetic rates. Consequently, the study of catch-up growth must be considered for each clinical state.

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TABLE III-1. Protein turnover in tibialis anterior during catch-up growth

| | d 4 | d 6 | d 8 | d 11 | d 14 | rEMS | P[1] |
|---|---|---|---|---|---|---|---|
| GROWTH (μg Phe/day) | | | | | | | |
| Control | 89.7 | 93.5 | 85.1 | 80.1 | 56.5 | 18.8 | <0.001 |
| Infected | 144.2* | 120.8* | 109.0* | 92.6* | 67.7 | | txd |
| $k_{growth}$ (%/day) | | | | | | | |
| Control | 6.6 | 5.9 | 4.8 | 4.2 | 2.6 | 1.4 | <0.001 |
| Infected | 12.7* | 9.5* | 6.9* | 5.0 | 3.2 | | txd |
| SYN (μg Phe/day) | | | | | | | |
| Control | 203.6 | 210.4 | 208.4 | 216.2 | 197.1 | 37.4 | NS |
| Infected | 207.4 | 208.1 | 223.7 | 241.5 | 195.5 | | |
| $k_{syn}$ (%/day) | | | | | | | |
| Control | 14.3 | 13.2 | 12.0 | 10.8 | 9.0 | 1.6 | <0.001 |
| Infected | 17.9* | 15.9* | 14.1* | 13.0* | 9.3 | | |
| DEG (μg Phe/day) | | | | | | | |
| Control | 113.9 | 119.8 | 128.1 | 140.5 | 140.6 | 32.8 | <0.05 |
| Infected | 57.1* | 94.0 | 114.4 | 143.5 | 127.8 | | |
| $k_{deg}$ (%/day) | | | | | | | |
| Control | 8.0 | 7.5 | 7.4 | 7.0 | 6.4 | 1.8 | NS |
| Infected | 4.7* | 6.9 | 7.2 | 7.7 | 6.1 | | txd |
| RNA (μg RNA-P/muscle) | | | | | | | |
| Control | 28.6 | 34.8 | 31.7 | 32.9 | 33.6 | 3.4 | NS |
| Infected | 27.1 | 31.5 | 34.8 | 35.6 | 35.3 | | |
| $C_s$ (μg RNA-P/mg Phe) | | | | | | | |
| Control | 20.9 | 21.3 | 18.3 | 18.4 | 15.4 | 2.1 | <0.001 |
| Infected | 23.3 | 26.6* | 22.8* | 20.4 | 16.8 | | |
| $k_{RNA}$ (μg Phe/μg RNA-P/day) | | | | | | | |
| Control | 6.09 | 6.30 | 6.24 | 5.38 | 5.87 | 0.64 | NS |
| Infected | 6.76 | 5.86 | 6.04 | 5.97 | 5.52 | | |

n = 3 to 12 animals per group

Phe mass at day 4 was 1.15 ± 0.05 mg (infected) vs. 1.41 ± 0.05 mg (control)

rEMS root Error Mean Square

[1] Probability of an overall treatment effect; txd Significant (P<0.05) treatmentxday interaction; * Different from control (P<0.05); differences betweeen individual means were calculated using t-tests

TABLE III-2. Protein turnover in gastrocnemius during catch-up growth

| | d 4 | d 6 | d 8 | d 11 | d 14 | rEMS | P[1] |
|---|---|---|---|---|---|---|---|
| GROWTH (μg Phe/day) | | | | | | | |
| Control | 290.9 | 303.4 | 276.9 | 260.0 | 180.5 | 59.4 | <0.001 |
| Infected | 404.4* | 336.5 | 301.9 | 259.7 | 182.4 | | txd |
| $k_{growth}$ (%/day) | | | | | | | |
| Control | 7.4 | 6.9 | 5.5 | 4.4 | 3.1 | 1.7 | <0.001 |
| Infected | 13.3* | 8.8 | 7.1 | 5.0 | 3.2 | | txd |
| SYN (μg Phe/day) | | | | | | | |
| Control | 604.5 | 642.2 | 689.9 | 719.9 | 616.6 | 82.4 | NS |
| Infected | 567.2 | 708.1 | 688.6 | 780.9 | 625.5 | | |
| $k_{syn}$ (%/day) | | | | | | | |
| Control | 15.4 | 14.8 | 13.6 | 12.1 | 10.4 | 1.6 | <0.001 |
| Infected | 18.2* | 18.3* | 15.9* | 15.1* | 11.0 | | |
| DEG (μg Phe/day) | | | | | | | |
| Control | 313.5 | 349.1 | 430.5 | 473.9 | 436.1 | 84.9 | NS |
| Infected | 168.2* | 394.1 | 386.2 | 504.5 | 443.1 | | txd |
| $k_{deg}$ (%/day) | | | | | | | |
| Control | 8.0 | 8.0 | 8.5 | 8.0 | 7.3 | 1.8 | NS |
| Infected | 5.2* | 9.8 | 8.9 | 9.6 | 7.8 | | txd |
| RNA (μg RNA-P/muscle) | | | | | | | |
| Control | 83.6 | 92.7 | 103.3 | 100.9 | 109.9 | 2.6 | NS |
| Infected | 76.4 | 91.3 | 95.6 | 105.8 | 111.0 | | |
| $C_s$ (μg RNA-P/mg Phe) | | | | | | | |
| Control | 21.4 | 21.9 | 20.1 | 17.7 | 18.6 | 2.6 | <0.05 |
| Infected | 23.2 | 23.8 | 22.5 | 20.3 | 19.4 | | |
| $k_{RNA}$ (μg Phe/μg RNA-P/day) | | | | | | | |
| Control | 7.18 | 6.44 | 6.80 | 7.27 | 5.64 | 0.90 | NS |
| Infected | 7.18 | 7.60 | 6.97 | 6.25 | 5.71 | | |

n = 3 to 12 animals per group

Phe mass on day 4 was 3.17 ± 0.13 mg (infected) vs. 3.93 ± 0.11 mg (control)

rEMS root Error Mean Square

[1] Probability of an overall treatment effect; txd Significant ($P<0.05$) treatmentxday interaction; * Different from control ($P<0.05$); differences betweeen individual means were calculated using t-tests

TABLE III-3. In vitro muscle protein degradation[1]

| | EDL | | | Epi |
|---|---|---|---|---|
| DEG | ($\mu$g Phe releasedx3 hours$^{-1}$xmuscle$^{-1}$) | | | |
| addition | -insulin | +insulin | %decrease | +insulin |
| Control | 2.54 | 2.31 | -9.1# | 1.11 |
| Infected | 2.18* | 1.93* | -11.5# | 0.99* |
| SEM | 0.08 | 0.08 | | 0.06 |
| $k_{deg}$ | | (%/day) | | |
| Control | - | 5.74 | - | 6.94 |
| Infected | - | 5.47 | - | 6.92 |
| SEM | - | 0.28 | - | 0.46 |

[1] Measured 4 days after infection

EDL extensor digitorum longus

Epi epitrochearis

n = 10 rats per treatment

* P<0.05 control vs. infected

# P<0.05 +insulin vs. -insulin

TABLE III-4. Protein turnover in skin during catch-up growth

| | d 4 | d 6 | d 8 | d 11 | rEMS | P[1] |
|---|---|---|---|---|---|---|
| GROWTH (g protein/day) | | | | | | |
| Control | 0.22 | 0.24 | 0.22 | 0.20 | 0.04 | <0.001 |
| Infected | 0.43* | 0.37* | 0.31* | 0.26* | | txd |
| $k_{growth}$ (%/day) | | | | | | |
| Control | 6.9 | 5.4 | 4.3 | 3.7 | 1.8 | <0.001 |
| Infected | 12.6* | 10.3* | 6.8 | 5.4 | | |
| SYN (g protein/day) | | | | | | |
| Control | 1.81 | 1.92 | 2.17 | 2.28 | 0.32 | NS |
| Infected | 1.57 | 1.89 | 1.81 | 2.11 | | |
| $k_{syn}$ (%/day) | | | | | | |
| Control | 47.5 | 48.4 | 41.4 | 40.7 | 7.4 | NS |
| Infected | 51.2 | 49.8 | 41.4 | 41.3 | | |
| DEG (g protein/day) | | | | | | |
| Control | 1.54 | 1.70 | 1.96 | 2.07 | 0.34 | <0.05 |
| Infected | 1.20 | 1.55 | 1.52 | 1.83 | | |
| $k_{deg}$ (%/day) | | | | | | |
| Control | 38.7 | 42.9 | 37.4 | 37.0 | 8.9 | NS |
| Infected | 38.6 | 40.6 | 34.8 | 35.6 | | |

n = 3 to 6 animals per group

Protein mass on day 4 was 3.04 ± 0.18 g (infected) vs. 3.97 ± 0.18 g (control)

rEMS root Error Mean Square

[1] Probability of an overall treatment effect; * Different from control (P<0.05); txd Significant (P<0.05) treatmentxday interaction; differences betweeen individual means were calculated using t-tests

TABLE III-5. Protein turnover in liver during catch-up growth

| | d 4 | d 6 | d 8 | d 11 | d 14 | rEMS | P[1] |
|---|---|---|---|---|---|---|---|
| GROWTH (mg Phe/day) | | | | | | | |
| Control | 2.2 | 2.3 | 2.1 | 1.9 | 1.4 | 0.48 | NS |
| Infected | 2.9 | 2.5 | 2.2 | 1.9 | 1.3 | | |
| $k_{growth}$ (%/day) | | | | | | | |
| Control | 5.4 | 5.1 | 3.9 | 3.7 | 2.4 | 0.70 | <0.05 |
| Infected | 6.9* | 5.4 | 4.4 | 3.4 | 2.5 | | txd |
| SYN (mg Phe/day) | | | | | | | |
| Control | 44.9 | 45.7 | 48.4 | 51.3 | 56.4 | 12.8 | NS |
| Infected | 56.5 | 48.6 | 52.1 | 68.6 | 47.5 | | |
| $k_{syn}$ (%/day) | | | | | | | |
| Control | 111.8 | 106.3 | 97.2 | 107.5 | 95.7 | 13.6 | NS |
| Infected | 123.9 | 109.8 | 104.3 | 111.3 | 91.8 | | |
| RNA (mg RNA-P/liver) | | | | | | | |
| Control | 3.63 | 3.83 | 4.20 | 4.75 | 4.70 | 0.48 | NS |
| Infected | 4.02 | 4.24 | 4.66 | 4.81 | 4.79 | | |
| $C_s$ ($\mu$g RNA-P/mg Phe) | | | | | | | |
| Control | 101.7 | 103.7 | 96.8 | 106.2 | 80.0 | 2.6 | NS |
| Infected | 112.1 | 102.2 | 102.2 | 103.2 | 92.7 | | |
| $k_{RNA}$ (mg Phe/mg RNA-P/day) | | | | | | | |
| Control | 10.4 | 9.9 | 9.8 | 9.8 | 12.0 | 1.2 | NS |
| Infected | 9.1 | 10.3 | 9.9 | 8.6 | 9.9* | | |

n = 3 to 12 animals per group

Phe mass on d 4 was 43.0 ± 2.3 mg (infected) vs. 39.9 ± 2.9 mg (control)

rEMS root Error Mean Square

[1] Probability of an overall treatment effect; txd Significant ($P<0.05$) treatmentxday interaction; * Different from control ($P<0.05$); differences betweeen individual means were calculated using t-tests

TABLE III-6. Protein synthesis in small intestine during catch-up growth

| | d 4 | d 6 | d 8 | d 11 | d 14 | rEMS | P[1] |
|---|---|---|---|---|---|---|---|
| $k_{syn}$ (%/day) | | | | | | | |
| Control | 101.3 | 83.0 | 84.9 | 77.2 | 78.9 | 12.3 | <0.001 |
| Infected | 114.4* | 94.3* | 92.1 | 93.2* | 77.1 | | |
| $C_s$ (μg RNA-P/mg Phe) | | | | | | | |
| Control | 132.0 | 113.9 | 95.4 | 120.1 | 116.4 | 21.9 | NS |
| Infected | 141.0 | 123.9 | 130.0 | 124.5 | 118.0 | | |
| $k_{RNA}$ (μg Phe/μg RNA-P/day) | | | | | | | |
| Control | 7.48 | 6.91 | 6.78 | 6.51 | 7.37 | 0.90 | NS |
| Infected | 7.42 | 7.30 | 6.82 | 6.78 | 6.55 | | |

n = 3 to 12 per group

Protein mass was 0.682 ± 0.029 g (infected) vs. 0.661 ± 0.018 g (control)

rEMS root Error Mean Square

[1] Probability of an overall treatment effect; txd Significant ($P<0.05$) treatmentxday interaction; * Different from control ($P<0.05$); differences betweeen individual means were calculated using t-tests

# IV. GENERAL DISCUSSION AND CONCLUSIONS

## A. GENERAL CONCLUSIONS

The focus of this research was to study whole body and tissue protein metabolism during catch-up growth following infection in weanling rats. This subject, as discussed in Chapter I, has been under researched, despite clear evidence that infection is a major problem in human health and in agriculture. This thesis was undertaken to begin to close this gap in our knowledge. The major conclusions of this research are summarized as follows:

1. E. coli peritonitis proved to be a simple and practical model of infection that produced a marked and reproducible effect on growth and whole body and tissue nitrogen status.

2. Complete catch-up growth, as measured by body weight, nitrogen balance, and tissue protein mass occurred within 3 weeks after infection.

3. On a whole body basis, catch-up growth was achieved by augmenting nitrogen balance by increasing apparent nitrogen digestibility, whilst nitrogen intake and urinary excretion remained unchanged.

4. Catch-up growth in small intestine was complete before that of other tissues, suggesting a high priority for protein repletion in this tissue.

5. Liver protein mass and turnover were not different between control and infected rats at any time point studied.

6. Catch-up growth in skeletal muscle and skin was achieved by decreasing the absolute rate of protein degradation. Absolute

rates of protein synthesis were not different between control and infected rats at any time point. Protein synthesis in muscle was maintained because RNA mass was maintained. This mechanism appears to be unique because accelerated rates of growth are usually associated with increased rates of protein turnover.

The information described in this thesis only begins to explain how catch-up growth occurred after infection. The following discussion describes the adaptations, hormones and factors, and mechanisms that may be involved in catch-up growth, and suggests experimental approaches to further clarify their nature.

## B. METABOLIC ADAPTATIONS PROMOTING CATCH-UP GROWTH

Based on the observations made in this thesis, three defined mechanisms contributed to catch-up growth following infection: 1) increased availibility of amino acids to the whole body, 2) differential tissue distribution of amino acids, and 3) increased efficiency of protein and amino acid use for protein accretion.

### Availability of Amino Acids

The gastrointestinal tract played a focal role in whole body protein repletion by increasing apparent nitrogen digestibility, thus making more amino acids available to the body. This observation is in contrast to catch-up growth following nutrient deprivation where increased food intake has been noted (Wilson and Osbourn 1960). The mechanism by which apparent digestibility was increased is not known, but this effect may have occurred through an increase in transit time

or permeability of the small intestine, or decreased endogenous nitrogen secretion. Another possibility is that apparent digestibility increased because the protein mass or absorptive surface or density of transporters of the small intestine increased beyond that of controls. There is evidence from other systems that the effective absorptive surface area of the small intestine can increase beyond that of healthy fed control animals. Increased brush border height and surface area have been observed in undernourished rats and rabbits, compared to full fed control animals (Buret et al. 1990, Curtis et al. 1990). Morphological studies, measuring villus crypt height and number as well as brush border surface area in animals recovering from infection would clarify this point. Further experiments suggested this response may be mediated by epidermal growth factor (Opleta-Madsen et al. 1991). Increased absorptive surface area and number of amino acid transporters have been observed following resection of the small intestine in rats; this occurred without any apparent qualitative changes in transporters (Karasov and Diamond 1987).

Allocation of Amino Acids

The time course and magnitude of protein repletion were tissue specific during catch-up growth. Protein mass of liver was not altered so no catch-up occurred. Protein mass of small intestine was restored before d 4 and that of skin and muscle beyond d 4. Protein repletion was particularly rapid early in catch-up growth in muscle and skin. The rapid protein repletion of small intestine may have been due to the abundant supply of amino acids that came in contact

with this tissue when appetite resumed. Amino acids may be differentially distributed to muscle and skin by altering the transport of amino acids into the cells. Evidence suggests there must have been increased amino acid transport into muscle and skin. Net protein synthesis requires exogenenously supplied amino acids. In addition, tissue protein mass of infected rats was smaller, therefore more amino acids must have been taken up into a tissue that had a smaller cellular mass. This would have required either increased activity or numbers of transporters compared to control rats. Failure to increase the uptake of amino acids could limit tissue protein repletion. Studies measuring amino acid uptake and transporter number and activity in tissues would be required to determine how these are affected during catch-up growth.

Efficiency of Protein and Amino Acid Use

Catch-up growth of tissues may be promoted by efficiently utilizing proteins and amino acids. During catch-up growth, this was accomplished by decreasing protein degradation in muscle and skin. In doing so the efficiency of protein accretion (protein gain/protein synthesis) increased. For example, on d 4 efficiency of protein accretion in tibialis anterior was higher in infected (70%) compared with control (46%) rats. It would be interesting to know how much degradation could decrease and efficiency of protein accretion could increase in this and other circumstances, and their exact time course. Measures of degradation used in this thesis and in Jepson et al. (1986) measured degradation over several days and are therefore insensitive to rapid changes.

Amino acids may be utilized more efficiently by decreased amino acid oxidation. Oxidative metabolism of free amino acids constitutes the main source of irreversible nitrogen loss from the whole body. On a whole-body basis there were no differences in the efficiency of amino acid utilization between treatments. However, there may be differences in individual tissues. It would be interesting to determine the rate of oxidation of amino acids in tissues during catch-up growth because sparing of amino acids would constitute an increased efficiency of amino acid use.

## C. FACTORS SIGNALLING TRANSITION FROM A CATABOLIC TO AN ANABOLIC STATE

### Factors Reducing Protein Degradation

Protein repletion of skeletal muscle and skin occurred by decreasing protein degradation. This may be mediated by circulating hormones, local factors, tissue sensitivity to these compounds or all of the above. The changes in these factors must occur rapidly and profoundly because of the rapid transition from increased to decreased degradation compared to normal healthy control animals. There may be increases in the circulating concentrations or local production of anabolic factors or decreases in catabolic factors. Furthermore, it is clear that different tissues catch-up at different rates, indicating there must either be differential sensitivities to circulating hormones and/or there is differential expression of local factors.

There is no information about which factors decrease protein degradation during catch-up growth. There are studies on factors

that mediate increased protein degradation in the catabolic phase of infection. (Beisel 1984, Kettlehut et al. 1988). However, these data do not generally extend into the catch-up phase. A variety of anabolic (insulin, growth hormone and insulin-like growth factor (IGF-I)) and catabolic factors (cortisol and thyroid hormones) may be considered. Insulin, IGF-I, and growth hormone are classical anabolic factors. Insulin has been shown to decrease muscle protein degradation (Kettlehut et al. 1988). However, data from Chapter III indicated there were no differences in the insulin-stimulated decrease in protein degradation between treatments. Plasma concentrations of insulin were not altered during the initial recovery period following endotoxin treatment, during which protein degradation was decreased (Jepson et al. 1986). These data suggest that neither circulating concentrations of insulin nor muscle sensitivity to insulin mediate the decrease in protein degradation.

High circulating concentrations of growth hormone are associated with high rates of growth (Pell and Bates 1990). The anabolic actions of growth hormone are likely mediated through IGF-I (Pell and Bates 1990). Growth hormone has been established as a primary regulator of IGF-I gene expression in liver and other extrahepatic tissues, including muscle (Turner et al. 1988, Sara and Hall 1990). Hepatic IGF-I is primarily released into the circulation, thus, IGF-I has both autocrine and paracrine effects (Sara and Hall 1990). IGF-I, like insulin, has been shown to decrease muscle protein degradation (Kettlehut et al. 1988). Muscle IGF-I gene expression has been shown to increase in a variety of anabolic conditions, including regeneration of skeletal muscle after injury (Skotter et

al. 1987) and compensatory growth of the kidney after unilateral phrenectomy (Fagan and Melmed 1987). In the latter case, IGF-I mRNA expression increased five fold within 24 hours of phrenectomy (Fagan and Melmed 1987). In addition, there were no changes in serum growth hormone or IGF-I concentrations. Increased local IGF-I expression may also be an important factor in tissue catch-up following infection, particularly since its local expression can potentially contribute to the differential effects at the tissue level.

Thyroid hormones and glucocorticoids are the classical catabolic hormones. Glucocorticoids generally increase protein degradation, however, adrenalectomy does not decrease basal protein degradation (Kettlehut et al. 1988). Although, low levels of thyroid hormones result in decreased basal protein degradation (Kettlehut et al. 1988), this may not be an important factor in catch-up growth because Jepson et al. (1986) demonstrated that the transition from increased to decreased muscle protein degradation in rats recovering from endotoxin was not associated with any changes in free or total triiodothyronine concentrations.

During infection, the acute phase response is associated with the production of factors by activated mononuclear phagocytes. Partially purified supernatants of activated monocytes increase muscle protein degradation <u>in vitro</u> (Baracos et al. 1983, Baracos 1985, Goldberg et al. 1988). Subsequent research has not yet identified the active factor(s) within this mixture.

The hormonal influences on protein degradation and proteolytic systems are complex. Available data do not readily suggest which signals mediate the activation and subsequent supression of muscle

proteolysis after infection. While the data do not single out any one hormone that may fill such a role, the answer may lie in the interaction and balance among these hormones and tissue sensitivities to these factors. For example, corticosterone causes insulin resistance (Odedra et al. 1982) and growth hormone action requires adequate levels of nutrients and insulin (Pell and Bates 1990). To address these questions, an integrated approach involving the measurement of the time course of anabolic and catabolic factors and tissue sensitivity to these hormones is needed over the entire duration of the catabolic and anabolic phases of infection.

## D. NUTRITIONAL AND DRUG STRATEGIES TO PROMOTE CATCH-UP GROWTH

Nutritional and/or drug strategies could be used to promote tissue protein repletion, by promoting amino acid absorption, increasing tissue protein synthesis, or further decreasing degradation. Early restoration of protein mass of the small intestine and increased nitrogen digestibility appear to be crucial, therefore, any factors promoting these would be beneficial. The first element in catch-up growth was the restoration of voluntary food intake and increased nitrogen digestibility. The prompt restoration of enteral foods is obviously required. Increasing the protein content of the diet during catch-up growth may be a further means of exploiting this phase to maximal extent. The composition of dietary proteins may also have an impact on catch-up growth if the amino acid needs for protein repletion deviate significantly from those needed for normal growth. For example, during the catabolic phase of infection, glutamine pools are depleted (Stehle et al.

1989); this has been associated with depressed protein synthesis and activated muscle protein breakdown (MacLennan et al. 1987, 1988; Wu and Thompson 1990), and because this amino acid is a preferred oxidative fuel for the small intestine, intestinal atrophy and reduced villus height (Ameh 1991). Early supplementation of this amino acid may assist in reversal of these changes to permit faster catch-up growth through early improvement of gastrointestinal function and muscle protein sparing. Clinically, glutamine (given as a dipeptide), has been successfully used to improve nitrogen balance in surgical and burn patients (Stehle et al. 1989).

The idea of treatment with anabolic agents as an adjunct to clinical nutrition has received attention recently. For example, in catabolic tumour-bearing rats treated with the anabolic ß-adrenergic agent clenbuterol and total parenteral nutrition, Chance et al. (1991) have demonstrated improved muscle protein mass. $ß_2$-adrenergic agents have been shown to increase protein mass by increasing RNA mass and protein synthesis (Maltin et al. 1987, Yang and McElliott 1989) and by decreasing protein degradation (Reeds et al. 1986, Yang and McElliott 1989). These agents proved to be particularily effective in limiting the decrease in protein synthesis and RNA mass and increase in degradation following denervation atrophy (Maltin et al. 1987). These agents potentially could also be used to promote tissue protein repletion during catch-up growth.

## E. CONCLUSIONS

In conclusion, the experimental findings in this thesis provide some of the first insight into the events that lead to tissue and

whole body protein repletion after infection. Further studies could focus on individual tissues or the whole body, regulatory events, cellular and molecular mechanisms, or on clinical treatments. Hopefully, this and future research will eventually lead to the development of either nutritional and/or drug strategies to promote catch-up growth.

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**APPENDIX 1A: Body weight of rats infected with *E. coli*[1]**
data are from Chapter II

| Day | Rep 1 Control (n=11)[2] | Rep 1 Infected (n=11)[3] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | (g/rat) | | | | | | | |
| -5 | 57.9 | 59.6 | 58.0 | 59.3 | NS[5] | NS | NS | 13.3 |
| -4 | 63.3 | 64.6 | 63.3 | 64.7 | NS | NS | NS | 15.1 |
| -3 | 69.2 | 70.7 | 68.4 | 70.0 | NS | NS | NS | 20.1 |
| -1 | 79.7 | 81.3 | 80.2 | 81.3 | NS | NS | NS | 29.3 |
| 0 | 85.1 | 86.3 | 86.0 | 86.9 | NS | NS | NS | 34.3 |
| 1 | 91.0 | 81.5 | 91.4 | 79.7 | <0.0001 | NS | NS | 39.4 |
| 2 | 96.8 | 87.8 | 97.5 | 83.2 | <0.0001 | NS | NS | 53.4 |
| 4 | 108.7 | 104.3 | 109.4 | 96.5 | <0.001 | NS | NS | 84.1 |
| 7 | 123.7 | 121.6 | 125.4 | 114.1 | NS | NS | NS | 116.6 |
| 9 | 132.6 | 134.5 | 136.1 | 124.8 | NS | NS | NS | 139.7 |
| 11 | 140.6 | 143.9 | 144.6 | 136.3 | NS | NS | NS | 140.0 |
| 14 | 155.7 | 162.0 | 157.2 | 154.9 | NS | NS | NS | 177.6 |
| 18 | 164.1 | 171.2 | 166.7 | 165.1 | NS | NS | NS | 198.9 |
| 22 | 173.4 | 180.4 | 183.4 | 183.7 | NS | NS | NS | 190.7 |
| 25 | 183.8 | 192.4 | - | - | NS | - | - | 270.0 |
| 32 | 195.1 | 203.6 | - | - | NS | - | - | 349.3 |
| 39 | 206.8 | 212.8 | - | - | NS | - | - | 425.8 |
| 44 | 221.2 | 227.4 | - | - | NS | - | - | 424.2 |

[1]Animals were infected on day 0.
[2]n = 6 day 19 to day 44.
[3]n = 7 day 19 to day 44.
[4]EMS = error mean square.
[5]NS = not significant.

**APPENDIX 1B: Body weight as a percentage of day 0 body weight[1]**
data are from Chapter II

| Day | Rep 1 | | Rep 2 | | Treat | Rep | TreatxRep | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | Control (n=11)[2] | Infected (n=11)[3] | Control (n=15) | Infected (n=5) | P | P | P | |
| | | | (%) | | | | | |
| 0 | 100.0 | 100.0 | 100.0 | 100.0 | - | - | - | - |
| 1 | 107.0 | 94.4 | 106.3 | 91.8 | <0.0001 | <0.05 | NS[5] | 5.1 |
| 2 | 113.9 | 101.7 | 113.4 | 95.8 | <0.0001 | <0.05 | <0.05 | 15.0 |
| 4 | 127.9 | 120.8 | 127.2 | 111.3 | <0.0001 | <0.05 | NS | 42.0 |
| 7 | 145.4 | 140.9 | 145.9 | 131.5 | <0.001 | NS | NS | 64.1 |
| 9 | 156.0 | 155.9 | 158.3 | 143.9 | <0.01 | NS | <0.01 | 59.2 |
| 11 | 165.4 | 166.6 | 167.8 | 157.1 | NS | NS | NS | 72.8 |
| 14 | 183.1 | 187.6 | 183.1 | 178.4 | NS | NS | NS | 103.0 |
| 18 | 193.1 | 198.4 | 194.0 | 190.2 | NS | NS | NS | 125.5 |
| 22 | 201.1 | 207.6 | 213.5 | 211.6 | NS | NS | NS | 126.5 |
| 25 | 212.0 | 221.2 | - | - | NS | NS | NS | 125.5 |
| 32 | 226.2 | 234.0 | - | - | NS | - | - | 148.1 |
| 39 | 239.7 | 244.5 | - | - | NS | - | - | 173.0 |
| 44 | 255.9 | 261.5 | - | - | NS | - | - | 160.8 |

[1] Animals were infected on day 0.
[2] n = 6 day 19 to day 44.
[3] n = 7 day 19 to day 44.
[4] EMS = error mean square.
[5] NS = not significant.

**APPENDIX 1C: Daily food intake[1]**
data are from Chapter II

| Day | Rep 1 Control (n=11)[2] | Rep 1 Infected (n=11)[3] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | | | (g/rat/day) | | | | | |
| -6to-4 | 9.9 | 9.8 | 11.4 | 11.1 | NS[5] | <0.0001 | NS | 0.43 |
| -4to-2 | 10.4 | 10.0 | 11.9 | 11.4 | NS | <0.001 | NS | 1.61 |
| -2to 0 | 10.8 | 10.7 | 12.3 | 11.8 | NS | <0.0001 | NS | 0.53 |
| 0to 1 | 13.5 | 4.7 | 13.8 | 2.5 | <0.0001 | NS | <0.05 | 2.18 |
| 1to 2 | 12.2 | 8.1 | 14.7 | 7.6 | <0.0001 | NS | <0.05 | 2.76 |
| 2to 4 | 12.9 | 11.6 | 14.4 | 10.6 | <0.0001 | NS | <0.05 | 2.53 |
| 4to 6 | 14.7 | 14.2 | 15.3 | 13.6 | <0.05 | NS | NS | 1.85 |
| 6to 8 | 14.5 | 14.9 | 14.9 | 13.6 | NS | NS | NS | 2.24 |
| 8to10 | 15.6 | 14.7 | 15.2 | 15.1 | NS | NS | NS | 3.24 |
| 10to14 | 14.9 | 14.9 | 15.4 | 14.8 | NS | NS | NS | 1.92 |
| 14to18 | 15.4 | 15.2 | 15.0 | 15.1 | NS | NS | NS | 2.51 |
| 18to23 | - | - | 15.2 | 15.4 | NS | - | - | 1.66 |
| 18to25 | 14.8 | 14.9 | - | - | NS | - | - | 2.54 |
| 25to32 | 15.9 | 16.3 | - | - | NS | - | - | 3.92 |
| 32to39 | 15.6 | 15.1 | - | - | NS | - | - | 2.13 |
| 39to44 | 16.3 | 16.0 | - | - | NS | - | - | 3.30 |

[1]Animals were infected on day 0.
[2]n = 6 day 19 to day 44.
[3]n = 7 day 19 to day 44.
[4]EMS = error mean square.
[5]NS = not significant.

**APPENDIX 1D:** **Daily nitrogen balance**[1]
data are from Chapter II

| Day | Rep 1 Control (n=11)[2] | Rep 1 Infected (n=11)[3] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | | (g/rat/day) | | | | | | |
| -2to 0 | 0.213 | 0.224 | 0.226 | 0.234 | NS[5] | NS | NS | 0.001 |
| 0to 2 | 0.251 | 0.090 | 0.274 | 0.054 | <0.0001 | NS | <0.05 | 0.004 |
| 2to 4 | 0.237 | 0.224 | 0.257 | 0.194 | <0.01 | NS | NS | 0.004 |
| 4to 6 | 0.227 | 0.248 | 0.247 | 0.246 | NS | NS | NS | 0.003 |
| 6to 8 | 0.203 | 0.231 | 0.221 | 0.251 | NS | NS | NS | 0.005 |
| 8to10 | 0.204 | 0.221 | 0.217 | 0.257 | NS | NS | NS | 0.005 |
| 10to14 | 0.158 | 0.183 | 0.196 | 0.208 | NS | <0.01 | NS | 0.007 |
| 14to18 | 0.130 | 0.141 | 0.157 | 0.169 | NS | NS | NS | 0.008 |
| 18to23 | - | - | 0.145 | 0.162 | NS | - | - | 0.004 |
| 18to25 | 0.132 | 0.141 | - | - | NS | - | - | 0.004 |
| 25to32 | 0.181 | 0.165 | - | - | NS | - | - | 0.014 |
| 32to39 | 0.147 | 0.129 | - | - | NS | - | - | 0.005 |
| 39to44 | 0.160 | 0.151 | - | - | NS | - | - | 0.022 |

[1]Animals were infected on day 0.
[2]n = 6 day 19 to day 44.
[3]n = 7 day 19 to day 44.
[4]EMS = error mean square.
[5]NS = not significant.

**APPENDIX 1E: Cumulative nitrogen balance[1]**
data are from Chapter II

| Day | Rep 1 Control (n=11)[2] | Rep 1 Infected (n=11)[3] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | | | (g/rat) | | | | | |
| 0 | 0.426 | 0.453 | 0.448 | 0.469 | NS[5] | NS | NS | 0.002 |
| 2 | 0.928 | 0.632 | 0.996 | 0.577 | <0.0001 | NS | NS | 0.012 |
| 4 | 1.403 | 1.080 | 1.509 | 0.965 | <0.0001 | NS | NS | 0.032 |
| 6 | 1.857 | 1.575 | 2.003 | 1.456 | <0.0001 | NS | NS | 0.058 |
| 8 | 2.263 | 2.037 | 2.445 | 1.957 | <0.001 | NS | NS | 0.098 |
| 10 | 2.671 | 2.479 | 2.879 | 2.472 | <0.05 | NS | NS | 0.139 |
| 14 | 3.303 | 3.210 | 3.653 | 3.303 | NS | NS | NS | 0.203 |
| 18 | 3.852 | 3.776 | 4.263 | 3.978 | NS | NS | NS | 0.252 |
| 23 | - | - | 4.981 | 4.789 | NS | - | - | 0.544 |
| 25 | 4.689 | 4.727 | - | - | NS | - | - | 0.332 |
| 32 | 5.958 | 5.881 | - | - | NS | - | - | 0.677 |
| 39 | 6.986 | 6.785 | - | - | NS | - | - | 0.954 |
| 44 | 7.783 | 7.539 | - | - | NS | - | - | 1.621 |

[1]Animals were infected on day 0.
[2]n = 6 day 19 to day 44.
[3]n = 7 day 19 to day 44.
[4]EMS = error mean square.
[5]NS = not significant.

APPENDIX 1F: Apparent efficiency of nitrogen utilization[1,2] data are from Chapter II

| Day | Rep 1 Control (n=11)[3] | Rep 1 Infected (n=11)[4] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[5] |
|---|---|---|---|---|---|---|---|---|
| | | | (%) | | | | | |
| -2to 0 | 73.3[6] | 77.1[7] | 71.8 | 78.0 | NS[8] | NS | NS | 54.7 |
| 0to 2 | 77.4 | 52.9 | 74.4 | 52.7[9] | <0.0001 | NS | NS | 132.3 |
| 2to 4 | 74.4 | 71.4 | 74.8 | 66.8 | NS | NS | NS | 108.7 |
| 4to 6 | 65.7 | 68.0 | 67.3 | 69.7 | NS | NS | NS | 128.9 |
| 6to 8 | 59.6 | 58.7 | 63.6 | 68.1 | NS | NS | NS | 123.1 |
| 8to10 | 57.7 | 56.2 | 57.6 | 64.8 | NS | NS | NS | 104.1 |
| 10to14 | 48.4 | 50.0 | 57.7 | 58.1 | NS | NS | NS | 122.6 |
| 14to18 | 44.5 | 39.3 | 45.5[10] | 46.1 | NS | NS | NS | 112.4 |
| 18to23 | - | - | 40.7 | 43.1 | NS | - | - | 64.9 |
| 18to25 | 48.6 | 40.4 | - | - | NS | - | - | 137.8 |
| 25to32 | 46.9 | 39.6 | - | - | NS | - | - | 68.9 |
| 32to39 | 36.4 | 33.4 | - | - | NS | - | - | 52.7 |
| 39to44 | 37.8 | 35.9 | - | - | NS | - | - | 181.9 |

[1]Apparent efficiency of nitrogen utilization (%) = nitrogen retained/ nitrogen absorbed x 100 = nitrogen balance /(nitrogen intake - faecal nitrogen) x 100.
[2]Animals were infected on day 0.
[3]n = 6 day 19 to day 44.
[4]n = 7 day 19 to day 44.
[5]EMS = error mean square.
[6]n = 10.
[7]n = 9.
[8]NS = not significant.
[9]n = 4.
[10]n =14.

**APPENDIX 1G: Apparent nitrogen digestibility[1,2]**
data are from Chapter II

| Day | Rep 1 Control (n=11)[3] | Rep 1 Infected (n=11)[4] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[5] |
|---|---|---|---|---|---|---|---|---|
| | | | (%) | | | | | |
| -2to 0 | 84.5 | 86.7 | 77.6 | 78.8 | NS[6] | <0.0001 | NS | 14.4 |
| 0to 2 | 79.2 | 78.1 | 79.5 | 70.2 | <0.01 | <0.05 | <0.05 | 29.8 |
| 2to 4 | 78.5 | 84.2 | 73.7 | 78.9 | <0.01 | <0.05 | NS | 32.4 |
| 4to 6 | 75.2 | 80.5 | 74.5 | 77.7 | <0.05 | NS | NS | 27.3 |
| 6to 8 | 75.2 | 81.7 | 72.3 | 77.6 | <0.05 | NS | NS | 45.8 |
| 8to10 | 73.2 | 83.3 | 76.2 | 80.7 | <0.05 | NS | NS | 67.7 |
| 10to14 | 72.1 | 76.4 | 68.3 | 74.5 | <0.05 | NS | NS | 58.5 |
| 14to18 | 65.0 | 74.3 | 70.2 | 75.1 | <0.05 | NS | NS | 86.9 |
| 18to23 | - | - | 73.0 | 75.1 | NS | - | - | 39.6 |
| 18to25 | 60.6 | 74.1 | - | - | <0.05 | - | - | 74.2 |
| 25to32 | 75.7 | 79.4 | - | - | NS | - | - | 22.0 |
| 32to39 | 80.7 | 80.7 | - | - | NS | - | - | 11.2 |
| 39to44 | 80.2 | 81.4 | - | - | NS | - | - | 4.2 |

[1]Apparent nitrogen digestibility (%) = (nitrogen intake - faecal nitrogen)/ nitrogen intake x 100.
[2]Animals were infected on day 0.
[3]n = 6 day 19 to day 44.
[4]n = 7 day 19 to day 44.
[5]EMS = error mean square.
[6]NS = not significant.

**APPENDIX 1H: Daily faecal nitrogen excretion[1]**
data are from Chapter II

| Day | Rep 1 Control (n=11)[2] | Rep 1 Infected (n=11)[3] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | | | (g/rat/day) | | | | | |
| -2to 0 | 0.054 | 0.045[5] | 0.091 | 0.081 | NS[6] | <0.0001 | NS | 0.001 |
| 0to 2 | 0.085 | 0.044 | 0.096 | 0.043 | <0.0001 | NS | NS | 0.006 |
| 2to 4 | 0.088 | 0.058 | 0.123 | 0.070 | <0.0001 | <0.01 | NS | 0.001 |
| 4to 6 | 0.116 | 0.089 | 0.126 | 0.100 | <0.005 | NS | NS | 0.001 |
| 6to 8 | 0.115 | 0.088 | 0.134 | 0.106 | <0.05 | NS | NS | 0.002 |
| 8to10 | 0.140 | 0.079 | 0.118 | 0.096 | <0.05 | NS | NS | 0.006 |
| 10to14 | 0.132 | 0.114 | 0.159 | 0.122 | <0.05 | NS | NS | 0.006 |
| 14to18 | 0.172 | 0.128 | 0.145 | 0.122 | NS | NS | NS | 0.010 |
| 18to23 | - | - | 0.167 | 0.157 | NS | - | - | 0.005 |
| 18to25 | 0.187[7] | 0.126 | - | - | NS | - | - | 0.017 |
| 25to32 | 0.124 | 0.110 | - | - | NS | - | - | 0.010 |
| 32to39 | 0.097 | 0.095 | - | - | NS | - | - | 0.004 |
| 39to44 | 0.104 | 0.095 | - | - | NS | - | - | 0.002 |

[1]Animals were infected on day 0.
[2]n = 6 day 19 to day 44.
[3]n = 7 day 19 to day 44.
[4]EMS = error mean square.
[5]n =10.
[6]NS = not significant.
[7]n = 5.

**APPENDIX II: Daily urinary nitrogen excretion[1]**
data are from Chapter II

| Day | Rep 1 Control (n=11)[2] | Rep 1 Infected (n=11)[3] | Rep 2 Control (n=15) | Rep 2 Infected (n=5) | Treat P | Rep P | TreatxRep P | EMS[4] |
|---|---|---|---|---|---|---|---|---|
| | | | (g/rat/day) | | | | | |
| -2to 0 | 0.080[5] | 0.069 | 0.088 | 0.068 | NS[6] | NS | NS | 0.001 |
| 0to 2 | 0.075 | 0.071 | 0.093 | 0.067 | NS | NS | NS | 0.002 |
| 2to 4 | 0.087 | 0.090 | 0.088 | 0.080 | NS | NS | NS | 0.003 |
| 4to 6 | 0.127 | 0.117 | 0.121 | 0.097 | NS | NS | NS | 0.004 |
| 6to 8 | 0.146 | 0.158 | 0.128 | 0.113 | NS | NS | NS | 0.004 |
| 8to10 | 0.155 | 0.170 | 0.157 | 0.114 | NS | NS | NS | 0.004 |
| 10to14 | 0.187 | 0.146 | 0.181 | 0.150 | NS | NS | NS | 0.012 |
| 14to18 | 0.192 | 0.218 | 0.190[7] | 0.200 | NS | NS | NS | 0.015 |
| 18to23 | - | - | 0.217 | 0.213 | NS | - | - | 0.013 |
| 18to25 | 0.151 | 0.211 | - | - | NS | - | - | 0.022 |
| 25to32 | 0.204 | 0.246 | - | - | <0.05 | - | - | 0.006 |
| 32to39 | 0.257 | 0.260 | - | - | NS | - | - | 0.009 |
| 39to44 | 0.257 | 0.265 | - | - | NS | - | - | 0.016 |

[1]Animals were infected on day 0.
[2]n = 6 day 19 to day 44.
[3]n = 7 day 19 to day 44.
[4]EMS = error mean square.
[5]n =10.
[6]NS = not significant.
[7]n = 14.

**APPENDIX 1J: Gastrocnemius nucleic acid analysis[1]**
data are from Chapter II

| | RNA | DNA | RNA/DNA |
|---|---|---|---|
| | mg/g tissue | mg/g tissue | |
| Control | 1.123(15) | 1.182(9) | 0.98(9) |
| Infected | 1.088(5) | 1.220(5) | 0.96(5) |
| SEM[2] | 0.070 | 0.175 | 0.19 |
| P | NS[3] | NS | NS |

Number of animals are shown in parentheses.
[1]Day 23; replicate 2.
[2]SEM - pooled standard error of the mean; differences between means were assessed using a Student's t-test at $P<0.05$.
[3]NS - not significant.

## APPENDIX 2: Preparation of *Escherichia coli*

*E. coli* were obtained from an outbreak of coliform septicaemia in turkey poults, and were provided by D. Onderka (Animal Health Division, Alberta Agriculture). The *E. coli* are of a single strain and have been serotyped (O78). Cell stocks were stored frozen in sheep blood at -50°C.

Bacteria were prepared 2 days prior to infection.

### Solutions

1. Tryptic soy broth

   To make 500 mL:

   30 g tryptic soy powder

   -add to 500 mL distilled water
   -autoclave 20 minutes

2. Tryptic soy agar

   To make 500 mL:

   30 g tryptic soy powder
   6 g agar

   -combine with 500 mL distilled water
   -autoclave 20 minutes
   -cool
   -while still warm pour in sterile petri dishes
   -store in refrigerator

   There is enough for 20 plates.

3. Phosphate buffered saline (pH 7.2)

   To make 2 L:

   0.28 g $NaH_2PO_4.H_2O$
   5.10 g $Na_2H_2PO_4$
   16.95 g NaCl

   -dissolve in 1.9 L water
   -adjust to pH 7.2 with 5 N HCl
   -bring volume to 1 L
   -autoclave 20 minutes

### Procedure

**Day 1**

The bacterial stock was taken out from the freezer and allowed to thaw slightly. A sterile toothpick was dipped into the blood and

smeared on two agar plates. Plates were incubated overnight at 37°C.

**Day 2**

Using a sterile toothpick, bacteria were scraped off the plates and put in two sterile screw-capped test tubes containing sterile tryptic soy both. The tubes were shaken and incubated 1 h at 37°C.

The contents of the tubes were placed in a sterile 500 mL volumetric flask containing about 300 mL of prewarmed tryptic soy broth. The solution was incubated at 37°C for 4 h while shaking. Dense growth appeared.

The bacteria were poured into two sterile centrifuge jars. They were centrifuged at 3000 g for 15 minutes. The supernatants were discarded and pellets combined in one jar. The bacteria were washed three times with about 100 mL sterile PBS. The bacteria were resuspended in about 100 mL PBS. The bacteria were stored at +4°C until used the next day.

The number of bacterial colony forming units (CFU) was determined. Dilutions, from $10^{-2}$ to $10^{-8}$ of the bacterial suspension were made. Dilutions were made in sterile 10 mL screw-capped test tubes. Sterile pipette tips were used. Tubes were shaken and vortexed between transfers.

The number of CFU was determined in quadruplicate from dilutions $10^{-6}$, $10^{-7}$, and $10^{-8}$. From these dilutions, 15 μL was plated on agar plates. Plates were inverted and incubated at 37°C for 8 to 16 hours.

**Day 3**

The number of colonies were counted and averaged. The number of colonies per mL of bacterial suspension was determined as follows:

CFU/mL = (colony count x dilution factor x 1000)/15

The bacteria were diluted with sterile PBS, such that in 0.5 mL there was $3.5 \times 10^9$ CFU/100 g body weight.

## APPENDIX 3: Nucleic acid analysis

### RNA analysis

RNA was analyzed by the method of Munro and Fleck (1969). This analysis was quantitative. Care was taken to not lose sample.

### 1. Homogenization

The objective of this step was to inactivate RNAases, and precipitate polymerized nucleic acids and proteins.

**Solutions**

1. 0.6 N perchloric acid ($HClO_4$)

   To make 1 L:

   51.6 mL 70% $HClO_4$

   -bring to 1 L with Milli Q (MQ) water

2. 0.2 N $HClO_4$

   To make 1 L:

   17.2 mL 70% $HClO_4$

   -bring to 1 L with MQ water

**Procedure**

Tissues were homogenized using a polytron homogenizer. Tibialis anterior, while frozen, was minced with a scalpel or fine scissors and homogenized on ice with 4 mL ice-cold MQ water in a 10 mL polypropylene test tube. Gastrocnemius, while frozen, was minced with a scalpel or fine scissors and homogenized on ice with 8 mL ice-cold MQ water in a 25 mL polypropylene test tube. About 0.2 to 0.25 g of frozen pulverized liver was homogenized on ice with 8 mL ice-cold MQ water in a 25 mL polypropylene test tube. About 0.5 to 1.0 g of small intestine (jejenum) was pulverized in liquid nitrogen with a mortar and pestle, then homogenized on ice in 8 mL ice-cold MQ water in a 25 mL polypropylene test tube. Tissues were homogenized for one minute until smooth. Five mL of ice-cold 0.2 N $HClO_4$ was added to the gastrocnemius and small intestinal homogenates. Two mL was added to tibialis anterior and liver. The homogenizer was rinsed two times with 2 mL 0.2 N $HClO_4$ for liver and tibialis anterior and with 5 mL for gastrocnemius and small intestine. Washings were combined with the homogenate.

Homogenates were centrifuged at 0°C and 3000 x g for 10 minutes. Supernatants were discarded. Pellets were washed twice with 5 mL 0.2 N $HClO_4$ and the washings discarded. The test tubes were inverted on paper towel to remove excess acid.

2. **RNA hydrolysis**

The objective of this step was to hydrolyze RNA and separate it from protein and DNA.

**Solutions**

1. 0.3 N Potassium Hydroxide (KOH)

   To make 1 L:

   19.4 g KOH

   -bring to 1 L with distilled water

2. 1.2 N $HClO_4$

   To make 1 L:

   103 mL 70% $HClO_4$

   -bring to 1 L with distilled water

3. 0.2 N $HClO_4$ (see above)

**Procedure**

To hydrolyze RNA, 4 mL of KOH was added to the tibialis anterior and liver pellets and 8 mL to the gastrocnemius and small intestinal pellets. Samples were floculated and vortexed at low speed. Tubes were placed in a shaking water bath at 37°C for 1 h. Tubes were floculated and vortexed every 15 minutes. At the of 1 h, tubes were placed in ice water. To precipitate protein and DNA, 2 mL of ice-cold 1.2 N $HClO_4$ was added to tibialis anterior and liver samples and 4 mL to gastrocnemius and small intestine. To ensure complete precipitation, tubes were kept on ice for 10 minutes. Samples were then homogenized at 0°C and 3000 g for 10 minutes. Samples were washed twice with 5 mL ice-cold 0.2 N $HClO_4$. Supernatant and washings were combined in a 100 mL volumetric flask. Ten mL 0.6 N $HClO_4$ was added. The volume was brought to 100 mL. Thus, the RNA is in 0.1 N $HClO_4$.

The pellet was saved for Phe or DNA analysis. See hydrolysis and DNA analysis below.

3. **Spectrophotometer**

The objective of this step was to quantitate the amount of RNA in each sample. This is done by measuring the absorption at 260 nm. Peptides also absorb at 260 nm causing interference. This is resolved by also measuring the absorption at 232 nm (Fleck and Begg 1965).

The concentration of RNA per mL final solution, measured as RNA-P(phosphorus) is calculated from the formula given by Fleck and Begg (1965):

Liver

$$C_{RNA\text{-}P} = (3.40 \times ABS_{260}) - (1.44 \times ABS_{232})$$
$$= \mu g \text{ RNA-P/mL}$$

Other tissues

$$C_{RNA\text{-}P} = (3.79 \times ABS_{260}) - (1.50 \times ABS_{232})$$
$$= \mu g \text{ RNA-P/mL}$$

The amount of RNA in the sample was determined by ultiplying the concentration of RNA by 100.

**Phe analysis**

When Phe was also being measured (for capacity of protein synthesis), the final pellet from RNA analysis was hydrolyzed to free amino acids and then analyzed for Phe. See Phe analysis (Appendix 5).

**DNA analysis**

DNA was analyzed by the Method of Munro and Fleck (1969). DNA was analyzed from the same sample as RNA. It was analyzed from the final pellet in the RNA analysis. Refer to RNA analysis. This analysis was quantitative. Care was taken to not lose sample.

**1. DNA hydrolysis**

The objective of this step was to solubilize and hydrolyze the DNA.

**Solutions**

1. 0.3 N KOH (see RNA analysis)
2. Concentrated HCl
3. HPLC grade chloroform
4. 0.04% indole

To make 1 L:

0.4 g indole

-dissolve indole in distilled water
-refrigerate

**Procedure**

Five mL of 0.3 N KOH was added to the final pellet from RNA analysis. The precipitate was left at room temperature or gently

warmed until dissolved. Twelve more mL of 0.3 N KOH was added. The volume was brought to 50 mL with distilled water. DNA was thus dissolved in 0.1 N KOH.

Two mL of this solution, 1 mL of indole solution, and 1 mL HCl were mixed. This mixture was placed in a boiling water bath for 10 minutes, and then cooled in running water. The solution was extracted three times with 4 mL chloroform. The chloroform layer settles to the bottom, and was discarded. The last extraction was centrifuged for 5 minutes at 500 g.

**2. Spectrophotometry**

The objective of this step was to quantitate the amount of DNA.

**Solutions**

1. DNA standard stock solution (0.4 μg/mL)

   To make 50 mL:

   20 mg salmon sperm DNA

   -mix with 50 mL distilled water
   -a little NaOH was required to dissolve DNA
   -store refrigerated

2. DNA working standard solution (16 μg/mL)

   -dilute stock solution 1 in 25

3. 0.1 N KOH

   To make 1 L:

   6.5 g KOH

   -dissolve KOH in distilled water

**Procedure**

To make the standard curve, 1, 1.5, 2, 3, 4, and 5 mL of the standard solution was added to 50 mL volumetric flasks, and brought to volume with 0.1 N KOH. Thus the concentration of the standards was 0.2, 0.3, 0.4, 0.6, 0.8, 1.0 μg/mL. Standards were boiled with HCl and indole, and extracted with chloroform, in the same way as the samples. Standards were done in duplicate.

Sample solutions and standards were read in a spectrophotometer at 490 nm. The standard curve was linear and samples fell within the range of the standard curve.

## REFERENCES

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Munro, H. N., and A. Fleck. Analysis of tissues and body fluids for nitrogenous constituents. In: Mammalian Protein Metabolism (vol. 3). New York: Academic, 1969, pp. 423-525.

APPENDIX 4: Effect of pair-feeding on tissue protein mass[1,2]

| Tissue | Control (n=7) | Pair-fed (n=7) | %Difference | SEM | P[3] |
|---|---|---|---|---|---|
| | (mg protein/tissue)[4] | | | | |
| Gastrocnemius | 88.5 | 86.4 | -2.4% | 2.9 | NS[5] |
| Tibialis anterior (left and right) | 55.9 | 53.6 | -4.1% | 2.2 | NS |
| Liver | 1068.9 | 996.4 | -6.7% | 61.3 | NS |
| Small intestine | 503.2 | 477.5 | -5.1% | 27.1 | NS |

[1]Pair-fed rats were fed, on a daily basis, the amount of food infected rats consumed, as a percent of control intake, over the first four days of infecton (Table II-2)
[2]Tissues were dissected on day 4
[3]Differences between means were assessed using t-tests
[4]Protein mass was analyzed using the Kjeldahl method (AOAC 1984)
[5]NS = Non-significant (P>0.05)

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**APPENDIX 5: Measurement of *in vivo* protein turnover**

**Fractional rate of protein synthesis**

**1. Injection of flooding-dose of ($^3$H)Phenylalanine (Phe)**

**Solutions**

1. Phosphate buffered saline (PBS) pH 7.4

To make 2 L:

16.9 g NaCl
5.1 g $Na_2HPO_4.H_2O$
0.28 g $NaH_2PO_4$

-dissolve reagents in 1900 mL MQ water
-adjust to pH 7.4 with 10 N NaOH
-bring volume to 2 L with Milli Q (MQ) water
-autoclave 20 minutes

2. ($^3$H)Phe solution

The final solution will contain 50 μCi/100 g body weight and 0 μmol Phe/100 g body weight, contained in 1.25 mL PBS/100 g body weight. Make up ~10% more solution than needed to account for dead space in syringes. This solution should not be made more than 1 to 2 days in advance.

2.065 g Phe ($C_9H_{11}NO_2$)
PBS
($^3$H)Phe (1mCi/mL)

-dissolve Phe in 100 mL PBS
-this solution will take ~1 hour to dissolve.

-draw out from isotope stock bottle 50 μL ($^3$H)Phe per 100 g body weight using a tuberculin seringe
-inject into an uncoated, sterile, and stoppered 10 to 15 mL vacutainer tube
-inject 1.2 mL of Phe solution per 100 g body weight through a 0.2 μ Millipore filter into the vacutainer tube
-mix and refrigerate

**Procedure**

On the day of the experiment, the animals were weighed and syringes of radioactive solution prepared. One rat was injected every 7 minutes. After precisely 15 minutes, the rat was killed by cervical dislocation. The rat was immersed in ice water and the visceral cavity opened. This will ensure that protein turnover is halted. It will also wash out any possible residual radioactivity in the visceral cavity. The carcass was kept in the ice bath for not less than one minute. During this time the

liver, gastrointestinal tract, and a 4 $cm^2$ piece of skin from the back were removed. The carcass was removed from the ice bath.

One person removed the tibialis anterior and gastrocnemius, and these muscles were frozen in liquid nitrogen, which were used to determine the fractional rate of protein synthesis. Muscles on the contralateral leg were dissected intact and frozen in liquid nitrogen; these muscles were used to determine Phe and RNA mass.

Another person froze the skin sample. Another person dissected a 10 to 15 cm section of small intestine (jejenum), rinsed it in ice cold saline, which was blotted, and frozen in liquid nitrogen. That person then dissected the liver which was blotted and frozen in liquid nitrogen. The carcasses were frozen in liquid nitrogen. Tissues were stored at -50°C.

**2. Tissue homogenization (when RNA is not being assayed)**

See Appendix 3 for tissue homogenization when RNA is assayed.

The objective of tissue homogenization was to separate free from protein-bound amino acids prior to determination of their specific radioactivities.

**Solutions**

1. 2% Perchloric acid ($HClO_4$)

To make 2 L:

-57 mL 70% $HClO_4$
-bring volume to 2 L with distilled water

**Procedure**

Tissues were homogenized using a motorized ground glass tissue homogenizer. Gastrocnemius and tibialis anterior and about 0.5 g of small intestine, while frozen, were minced with a scalpel or fine scissors. The entire liver was pulverized with liquid nitrogen in a mortar and pestle, and about 0.5 grams was used for analysis. Tissues were homogenized, on ice, at high speed with ice-cold 2% (w/v) $HClO_4$. Gastrocnemius, liver, and small intestine were homogenized with 3 mL $HClO_4$ and tibialis anterior with 2 mL $HClO_4$. They were homogenize until smooth, which took approximately one minute. Skin was more difficult to homogenize. Approximately 0.5 g of skin was minced, while frozen, with a scalpel, then pulverized with liquid nitrogen in a mortar and pestle. Skin was homogenized with 3 mL of $HClO_4$ until smooth, which took about 20 minutes. The homogenate was poured into a 15 mL screw capped Pyrex test tube. The homogenizer was rinsed two times with 0.5 mL 2% $HClO_4$ and washings combined with homogenate. Homogenates were stored on ice, until centrifuged.

The homogenate was centrifuged at 1000 x g for 15 minutes. The supernatant was decanted into a 10 mL test tube and saved for

analysis of intracellular Phe. The pellet was saved for analysis of protein-bound Phe.

3. **Intracellular fraction**

The objective of this step was to precipitate perchlorate ions and neutralize the solution for subsequent enzymic conversion.

**Solutions**

1. Saturated potassium citrate ($K_3C_6H_6H_5O_7$)

To make 50 mL:

70 g potassium citrate

-dissolve in 50 mL distilled water
-keep adding 5 g of potassium citrate until no more will dissolve
-gently heat until dissolved
-cool

**Procedure**

Half a volume of saturated potassium citrate was added to the intracellular fraction. A whitish crystalline precipitate formed. Samples were centrifuged at 2000 x g. The supernatant (intracellular fraction) was decanted into 10 mL test tubes and stored frozen. The precipitate was discarded.

4. **Protein-bound fraction**

The purpose of this step was to remove any free amino acids, then hydrolyse the protein-bound amino acids, remove the acid, and neutralize the solution for subsequent enzymic conversion.

**Solutions**

1. 2% $HClO_4$ (see above)

2. 6 N HCl

To make 1 L:

500 mL concentrated HCl
500 mL distilled water

-pour acid into water
-cool
-bubble with nitrogen

3. 0.5 M Tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) (pH 6.3)

To make 100 mL:

14.71 g tri-sodium citrate

-dissolve in 100 mL distilled water
-adjust to pH 6.3 with 10 M HCl

**Procedure**

The protein pellet was washed with 8 mL ice-cold 2% $HClO_4$ and centrifuged for 15 minutes at 3000 x g. The supernatant was discarded. This step was repeated twice.

Five mL 6 N HCl was added. It was important to make sure that the pyrex test tubes have no chips on lip (to prevent evaporation). Tubes were purged with nitrogen, capped with screw caps with teflon liners, and heated to 110°C for 24 hours. After 0.5 h caps were tightened and checked for evaporation, and tubes were vortexed. The solution turned black. When there was evaporation, lost volume was replaced with 6 N HCl.

Acid was then removed. It may be removed by freeze drying, nitrogen gas, or vacuum centrifuge (Speed-vac concentrator). Vacuum centrifugation at 65°C was preferred. The amino acids were resuspended in 0.5 mL sodium citrate (pH 6.3). The pH was adjusted to 6 to 7 using 10 N NaOH, and stored frozen. This solution is ready for enzymic conversion.

**5. Enzyme conversion**

The objective of this step was to convert Phe to ß-phenylethylamine (PEA) so that it could be separated from other amino acids, such as tyrosine and tyramine, that may have become labelled during the period of incorporation.

**Solutions**

1. 0.5 M sodium citrate (pH 6.3) (see above)

2. Enzyme suspension

This contains 2 Units/mL L-tyrosine decarboxylase (Sigma T4379), 1 mg/mL pyridoxal phosphate (Sigma P9255) in 0.5 mL 0.5 M sodium citrate (pH 6.3). Tyrosine decarboxylase can be used in place of Phe decarboxylase, because it is contaminated with the former. It is generally less costly than Phe decarboxylase.

To make 50 mL of enzyme suspension:

100 units tyrosine decarboxylase
0.05 g pyridoxal phosphate
0.5 M sodium citrate buffer (pH 6.3)

-combine ingredients

-the enzyme does not dissolve
-the suspension may be stored at -50°C
-mix well before using

**Procedure**

Half a mL of enzyme suspension was added to 1.0 mL of supernatant fraction or 0.5 mL of neutralized hydrolysate. The tubes were stoppered and incubated for 17 h in a shaking water bath at 50°C.

6. **Solvent extraction**

The purpose of this step was to separate the PEA from other amino acids. PEA was extracted by the method of Suzuki and Yagi (1976) as modified by McAllister (1987).

**Solutions**

1. 3 M NaOH

To make 100 mL:

12 g NaOH

-dissolve in 100 mL distilled water

2. 0.1 N $H_2SO_4$

To make 1 L:

2.8 mL concentrated sulphuric acid
-add to 1 L distilled water

3. Chloroform:n-heptane (1:3 v/v)

Use distilled reagent grade chloroform and hepane. Do not use HPLC grade chloroform.

**Procedure**

Half a mL of 3 M NaOH was added to the entire incubated enzyme solution. Amino acids become basic and polar; PEA becomes neutral and non-polar. Five mL chloroform-n-heptane was then added. Tubes were stoppered, shaken, and then centifuged at 500 g for 5 minutes. Amino acids, because they are polar remain in the aqueous phase; PEA because it is non-polar is extracted into the organic phase. The upper organic phase was carefully removed by pipette. Care was taken not to contaminate it with any of the bottom aqueous phase. Chloroform is more dense than water; the organic layer is on top because n-heptane, which is miscible in chloroform makes the organic solution less dense. The aqueous phase was discarded.

Five mL chloroform and 2 mL 0.1 N $H_2SO_4$ were added to the organic phase. The tubes were stoppered, shaken, and then

centrifuged at 500 g for 5 minutes. Under acidic conditions PEA is polar and is extracted into the aqueous phase. The top aqueous phase was removed by pipette. The organic layer settles to the bottom because the added chloroform makes the organic solution more dense. The organic layer was discarded. The 0.1 N $H_2SO_4$ containing PEA was left uncovered at room temperature to ensure all traces of chloroform were removed. Residual chloroform caused poor pipetting. Once pipetted, solutions were tightly covered.

7. **HPLC**

The purpose of this step was to measure the concentration of PEA in the final aqueous extract.

**Solutions**

All solutions were made up in glassware that was rinsed with HPLC grade water. Chemicals were always weighed on the same balance. Pipettes were calibrated before use. Great care was taken when pipetting.

1. 25 nmol/mL Ethanolamine (EA) ($C_2H_7NO.HCl$) (internal standard)

   To make 100 mL:

   0.0975 g EA

   -dissolve EA in 200 mL HPLC water
   -this stock solution (5 µmol/mL) may be used for 3 days

   -To make working solution:

   -combine exactly 0.500 mL of stock solution with 100 mL HPLC grade water
   -the working solution is made daily

2. 5 µmol/mL EA (internal standard)

   To make 200 mL:

   0.0975 g EA

   -dissolve EA in 200 mL HPLC grade water
   -refrigerate
   -this solution may be used for 3 days

3. 25 nmol/mL ß-Phenylethyamine ($C_8H_{11}N.HCl$) (PEA)

   To make 100 mL:

   0.1576 g PEA

   -dissolve PEA in 200 mL HPLC grade water

-refrigerate
-this stock solution (5 μmol/mL) may be used for 3 days

To make working solution:

-combine exactly 0.500 mL of stock solution with 100 mL HPLC grade water
-the working solution is made daily.

4. 5 μmol/mL PEA

0.1576 g PEA

To make 200 mL:

-dissolve PEA in 200 mL HPLC grade water
-refrigerate
-this solution may be used for 3 days

5. Buffer A

To make 2 L:

11.5 glacial acetic acid
8.0 g NaOH
180 mL HPLC grade methanol
10 mL tetrahydrafuran

-dissolve acid and base in 1600 mL HPLC grade water
-adjust to pH 7.2 with 5 M NaOH
-add methanol and tetrahydrofuran
-bring volume to 2 L with HPLC grade water

6. OPA solution

To make 250 mL:

1 g OPA (O-phthaldehyde Sigma P1378)
25 mL HPLC grade methanol
6 mL Brij 35
1 mL 2-mercaptoethanol
224 mL 0.4 M sodium borate buffer (see below)

-dissolve OPA in methanol in a dark or covered bottle
-add Brij 35, 2-mercaptoethanol, and 0.4 M sodium borate buffer
-refrigerate
-solution may be used for 1 or 2 weeks
-unrefridgerated OPA may be used for 1 day

7. 0.04 M Sodium borate buffer ($Na_2B_4O_7.(10H_2O)$) pH 9.5

To make 1 L:

15.234 g of sodium borate

-dissolve sodium borate in 900 mL HPLC grade water
-adjust pH to 9.5 with 5 M NaOH
-bring volume to 1 l

8. Saturated potassium borate

To make 1 L:

30 g potassium borate ($K_2B_4O_7.(4H_2O)$)

-add potassium borate to 1 L HPLC grade water
-mix
-add solute until no more will dissolve

9. Standard (for free PEA)

EA (25 nmol/mL)
PEA (25 nmol/mL)
saturated potassium borate buffer
0.1 N $H_2SO_4$
HPLC grade water

-combine in ashed or new HPLC vials in proportions of 1:1:2:1:8
-parafilm or cover with septa
-shake and vortex

These proportions may vary slightly depending upon how much PEA is in the samples.

10. Standard (for protein-bound PEA)

To make amino acid standard solution:

50 μL EA (5 μmol/mL) (see above)
50 μL PEA (5 μmol/mL) (see above)
2 mL HPLC grade water

-combine in a test tube
-vortex

To make standard:

amino acid standaard solution
saturated potassium borate buffer
0.1 N $H_2SO_4$
HPLC grade water

-combine in ashed or new HPLC vials in proportions of 1:4:1:16
-parafilm or cover with septa and cap
-shake and vortex

These proportions may vary slightly depending upon how much PEA is in the samples.

11. Free PEA sample

EA (25 nmol/mL)
final extract of sample
saturated potassium borate buffer
0.1 N $H_2SO_4$
HPLC grade water

-combine in ashed or new HPLC vials in proportions of 1:1:2:1:8
-parafilm or cover with septa and cap
-shake and vortex

These proportions may vary slightly depending upon how much PEA is in the samples.

12. Protein-bound PEA sample

To make sample amino acid solution:

50 µL sample
50 µL PEA (5 µmol/mL)
2 mL HPLC grade water

-combine in a test tube
-vortex

To make sample for injection:

sample amino acid solution
saturated potassium borate buffer
0.1 N $H_2SO_4$
HPLC grade water

-combine in ashed or new HPLC vials in proportions of 1:4:1:16
-parafilm or cover with septa and cap
-shake and vortex

These proportions may vary slightly depending upon how much PEA is in the samples.

**Procedure**

The HPLC consisted of a Varian liquid chromatograph coupled to a fluorochrome detector. The fluorimeter was set at an excitation range of 340-380 nm and an emission cutoff at 390 nm. PEA and EA were derivatized with OPA immediately before the sample was injected onto the column; this method was originally used for amino acids (Jones and Gilligan 1983). A C-18 reverse-phase column (Supelco) (75 x 4.6 mm I.D.; 40-60 µm

particle size) was used for all samples. Two solvents, solvent A and solvent B, methanol, were used to form the following gradient: 40% B at 0 minutes to 90% B at 4.5 minutes, 90% B from 4 minutes to 4.5 minutes, and 40% B from 4.5 minutes to 10.3 minutes. The flow rate was 1.5 mL/minute.

Each sample was done in duplicate. When the percent difference between duplicates was greater than 5%, it was repeated. One standard was used for every 10 samples. There must only be two main peaks. Other peaks indicates that the solvent extraction of PEA was incorrectly done. HPLC was done before liquid scintillation counting in case re-extraction was necessary; this will save sample.

**8. Liquid scintillation counting**

Pipettes were calibrated carefully. A volume of 200 to 500 uL of the final extract containing PEA in 0.1 N $H_2SO_4$ was pipetted into in a scintillation vial, depending on the number of DPM. At least 500 DPM was counted. Samples were done in duplicate. Five mL of scintillation fluid was added, vials capped, and vials shaken very well. Vortexing does not work. Samples were dark adapted for about 1 h and counted in a liquid scintillation counter for 20 minutes or until 10,000 counts accumulated ($\sigma$=0.05%). Samples were monitored for two phases, chemiluminescence, and photoluminescence. In practice this was not necessary. When the percent difference was greater than 5% between duplicates, the sample was repipetted. Differences were normally less than 2%.

**9. Specific radioactivity of Phe**

The specific radioactivity of Phe is equal to that of PEA, assuming the tritium atom on ($^3$H)Phe has no effect on its conversion to PEA. The specific radioactivity (SRA) of PEA is calculated by dividing the radioactivity of the final extract by the concentration of PEA in the final extract:

$$\text{SRA of PEA} = (\text{DPM/mL})/(\text{nmol/mL}) = \text{DPM/nmol}$$

**10. Calculation of fractional rate of protein synthesis**

The fractional rate of protein synthsis ($k_{syn}$) (%/day) for each issue was calculated from the formula of McNurlan et al. (1979) and Garlick et al. (1980):

$$k_{syn} = S_B/(S_A x t) x 100$$

where $S_B$ is the specific radioactivity of protein-bound Phe, $S_A$ is the specific radioactivity of tissue free Phe, and t is time of incorporation.

**Absolute rate of protein synthesis**

To calculate the absolute rate of protein synthesis, the Phe or protein mass of that tissue must be measured. We used Phe mass, because it has been suggested that it more correctly represents protein synthesis because it was the tracer used (Baracos et al. 1991). We used protein mass for skin because of difficulties in obtaining a homogeneous sample.

**1. Hydrolysis**

The objective of this step was to hydrolyse tissue proteins in order to measure protein-bound Phe mass.

Skeletal muscles and liver, which has been pulverized can be directly hydrolyzed; the protein does not have to be precipitated because free Phe represents less than 1% of total tissue Phe, even when a flooding-dose of Phe is used. Free Phe represents as much as 5% of the total tissue Phe in small intestine; tissue proteins should be precipitated. Do not use $HClO_4$

**Solutions**

1. 6 N HCl (see above)

**Procedure**

Entire muscles and 0.5 g pulverized liver were combined with 5 mL of 6 N HCl in a screw-capped Pyrex test tube. There should be not less than 5 mL acid per gram tissue. Samples were hydrolysed as described previously.

**2. HPLC**

The purpose of this step is to measure the Phe concentration in the hydrolysates.

All solutions were made up in glassware that was rinsed with HPLC grade water. Chemicals were always weighed on the same balance. Pipettes were calibrated before use. Great care was taken when pipetting.

**Solutions**

1. 100 µmol/mL ß-aminobutyric acid (baba) ($C_2H_7NO.HCl$) (internal standard)

To make 50 mL:

0.5155 g baba

-dissolve baba in 50 mL HPLC grade water
-refrigerate
-this solution may be used for 3 days

2. 100 μmol/mL Phe ($C_9H_{11}NO_2$)

   To make 100 mL:

   1.6520 g Phe

   -dissolve in 50 mL HPLC grade water
   -refrigerate
   -this solution may be used for 3 days

3. Buffer A (see above)

4. OPA solution (see above)

5. 0.04 M Sodium borate buffer (pH 9.5) (see above)

6. Saturated potassium borate buffer (see above)

7. Amino acid standard

   To make amino acid standard solution:

   100 to 200 μL Phe (100 μmol/mL)
   100 to 200 μL baba (100 μmol/mL)
   3 to 5 mL 6 N HCl

   -combine and mix

   The amount of amino acid should correspond to the amount of Phe in the sample.

   To make standard:

   amino acid standard solution
   saturated potassium borate buffer
   HPLC grade water

   -combine in ashed or new HPLC vials in proportions of 1:40:400
   -parafilm or cover with septa
   -shake and vortex

8. Hydrolysates

   To make sample solution:

   100 to 200 μL of baba (100umol/mL)
   hydrolysate

   -add baba into hydrolysate tube
   -cap, vortex, and shake

   To make injection solution:

sample solution
saturated potassium borate buffer
HPLC grade water

-combine in ashed or new HPLC vials in proportions of 1:40:400
-parafilm or cover with septa
-shake and vortex

These proportions depend on the amount of Phe present in the sample.

**Procedure**

Refer to previous section for a description of the system. Two solvents, solvent A and solvent B, methanol, were used to form the following gradient: 38% B at 0 minutes to 50% B at 6 minutes, 50% B from 6 minutes to 80% B 6.1 minutes, and 80% B from 6.1 minutes to 7 minutes. The flow rate was 1.5 mL/minute. During some runs, a peak co-eluted with Phe. To separate the peaks, following gradient was used: 35% B at time 0 to 42% B at 7.3 minutes, 42% B at 7.3 minutes to 80% B at 7.9 minutes, 80% B at 7.9 minutes to 8.9 minutes, and 80% B at 8.9 minutes to 35% B at 9 minutes.

Each sample was repeated twice. When the percent difference between duplicates was greater than 5% it was repeated.

3. **Protein Mass**

For the analysis of skin, rats were thawed and shaved using a scalpel blade and soap or electric clippers. The scalpel worked better. The rats were skinned. This procedure was arduous and took 45 minutes per rat. Skin also has less than 1% free Phe, so its protein did not have to be precipitated. To obtain a homogeneous sample of skin, the skin was autoclaved for a 2 h. This softened the skin, so it was easily homogenized. The skin homogenate was weighed. The protein content of skin was measured by the Kjeldahl method (AOAC 1984). The skin homogenate was mixed before sampling. About 0.5 g was used and was done in duplicate.

4. **Calculations**

The absolute rate of protein synthesis can be obtained by multiplying the fractional rate of protein synthesis by either the Phe or the protein mass of the tissue:

$$SYN = k_{syn} \times \text{Phe mass} = \mu g \text{ Phe/d} \quad \text{or} \quad SYN = k_{syn} \times \text{protein mass} = \text{mg protein/d}$$

**Capacity for protein synthesis**

The capacity for protein synthesis was calculated by dividing the RNA mass (see Appendix 3) by the Phe or protein mass.

**Efficiency of protein synthesis**

The efficiency of protein synthesis was calculated by dividing the absolute rate of protein synthesis by the RNA mass (see Appendix 3 for measurement of RNA mass).

**Fractional and absolute rates of growth**

Growth rates of tissues were determined from the Phe or protein masses on days 4, 6, 8, 11, and 14. Tissue Phe or protein mass was regressed against body mass for the entire group for each treatment. Body mass was regressed against day for each animal. The growth rate was obtaind by multiplying the slope of Phe or protein mass versus body mass by the slope of body mass versus day for each animal (Attaix and Arnal 1987).

**Fractional and absolute rates of protein degradation**

Rates of protein degradation were calculated as the difference between synthesis and growth.

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**APPENDIX 6: In vitro protein degradation**

Protein degradaton was determined in the extensor digitorum longus (EDL) and epitrochlearis (Epi). Total protein degradation was measured by the release of phenylalanine (Phe) from muscles in the presence of cycloheximide (Tischler et al. 1982).

**1. Muscle incubation**

**Solutions**

1. Krebs-Ringer bicarbonate (KRB) medium

NaCl stock solution (20 g/L)
$NaHCO_3$ stock solution (7 g/L)
KCl stock solution (3.6 g/100 mL)
$CaCl_2.2H_2O$ stock solution (1.47 g/100 mL)
$MgSO_4.7H_2O$ stock solution (3.08 g/100 mL)
$NaH_2PO_4$ (1.91 g/200 mL)
HEPES/NaOH stock solution (0.953 g/100 mL) pH 7.4
Glucose
Branched chain amino acid stock solution ((0.656 g leucine + 1.115 g isoleucine + 1.172 g valine)/100 mL)
Bovine insulin (25 U/mg)
Cycloheximide

To make 800 mL of KRB medium:

-branched chain amino acid stock solution is kept frozen
-other stock solutions are kept in the refrigerator and contain 30 µg/mL chloramphenicol to prevent bacterial growth

-on the day before or of the experiment, combine:
280 mL NaCl stock solution
240 mL $NaHCO_3$ solution
8 mL KCl stock solution
8 mL $CaCl_2$ stock solution
8 mL $MgSO_4$ stock solution
8 mL $NaH_2PO_4$ stock solution
40 mL HEPES/NaOH stock solution
200 mL Milli Q (MQ) $H_2O$

-on the day of the experiment, heat the above mixture to 35°C and gas with 95%$O_2$-5%$CO_2$ and adjust pH to 7.4 with NaOH
-add 0.721 g glucose
-add 8 mL branched chain amino acid stock solution
-add 0.1126 g cycloheximide
-dissolve 0.080g insulin in 1 mL KRB medium; a drop of 0.1 M NaOH will facilitate dissolution; add 100 µL of this solution to the KRB medium

2. 2 % Trichloracetic acid (TCA)

-dissolve 2 g TCA in 100 mL MQ $H_2O$

**Procedure**

Rats were killed by cervical dislocation, and EDL and Epi were rapidly dissected. EDL were mounted on stainless steel supports at their resting length *in situ*. Muscles were pre-incubated for 30 minutes in 3 mL Krebs-Ringer bicarbonate medium, composed of (in mM) 119 NaCl, 1.25 $MgSO_4$, 1.0 $CaCl_2$, 1.24 $NaH_2PO_4$, 25 $NaHCO_3$, 2.0 N-2-hydroxoethylpiperazine-N'-2-ethanesulphonic acid (HEPES)/NaOH at pH 7.4, 5.0 glucose, 0.85 leucine, 0.5 isoleucine, and 1.0 valine, plus 0.5 U insulin/mL. The medium was continually bubbled with 95%$O_2$-5%$CO_2$, and maintained at 35°C. The media also contained 0.5 mM cycloheximide to block reincorporation of Phe into tissue proteins. After 30 minutes muscles were transferred to fresh media of the same composition and incubated for 3 h. At the end of the 3 h incubation, muscles were removed, and placed in 1 mL of 2 % TCA; tissues and incubation media were stored frozen.

2. **Homogenization**

Muscles were homogenized to separate the free from protein bound amino acids.

**Solutions**

1. 2 % TCA (see above)

**Procedure**

Muscles were homogenized in 0.5 mL 2 % TCA using a motor-driven ground glass tissue homogenizer. Homogenates were placed in a 5 mL screw-capped test tube (no chips on lip). The homogenizer was rinsed two times with 0.5 mL 2 % TCA, and the washings combined. The homogenate was centrifuged at 3000 g for 15 minutes. The supernatant was frozen and saved for analysis of Phe (intracellular fraction).

3. **Hydrolysis**

The objective of this step was to hydrolyze muscle proteins in order to measure protein-bound Phe.

**Solutions**

1. 6 N HCl

To make 1 L:

500 mL concentrated HCl
500 mL distilled water

-pour acid into water
-cool
-bubble with nitrogen

2. 2% TCA (see above)

**Procedure**

Protein pellets were washed twice with 2 mL 2% TCA. Supernatants were discarded. One mL of 6 N HCl was added to the protein pellet. Tubes were capped with teflon lined lids, purged with nitrogen, and hydrolysed for 24 hours at 110°C. After 30 minutes, tubes were checked for evaporation, lids tightened, and vortexed. Hydrolysates were stored frozen until analyzed for Phe.

4. **HPLC**

The purpose of this step was to measure the concentration of Phe in the incubation medium, intracellular fraction, and the protein bound fraction (hydrolysate).

Refer to Appendix 5 for further details on HPLC

**Solutions**

1. 5 nmol/mL ß-amino butyric acid (baba) ($C_4H_{11}O_2$) (internal standard for incubation media and intracellular fraction)

   To make 100 mL:

   0.1031 g Baba

   -dissolve baba in 200 mL HPLC water
   -this stock solution (5umol/mL) may be used for 3 days

   To make working solution:

   -combine exactly 100 µL of stock solution with 100 mL HPLC grade water
   -the working solution is made daily

2. 10 µmol/mL baba (internal standard for protein bound fraction

   To make 100 mL:

   0.1031 g baba

   -dissolve baba in 100 mL HPLC water
   -this solution (10 µmol/mL) may be used for 3 days

3. 5 nmol/mL Phe ($C_9H_{11}NO_2$) (internal standard for incubation media and intracellular fraction)

   To make 100 mL:

   0.1652 g Phe

   -dissolve Phe in 200 mL HPLC water
   -this stock solution (5umol/mL) may be used for 3 days

   To make working solution:

   -combine exactly 100 µL of stock solution with 100 mL HPLC grade water
   -the working solution is made daily

4. 10 µmol/mL Phe (internal standard for protein bound fraction)

   To make 100 mL:

   0.1652 g Phe

   -dissolve Phe in 100 mL HPLC water
   -this solution (10 µmol/mL) may be used for 3 days

5. Buffer A (refer to Appendix 5)

6. OPA solution (refer to Appendix 5)

7. Saturated potassium borate buffer (refer to Appendix 5)

8. 2 % TCA (see above)

9. Standard for incubation medium

   baba (5 nmol/mL)
   Phe (5 nmol/mL)
   saturated potassium borate buffer
   HPLC grade water

   -combine in ashed HPLC vials in proportions of 2:2:2:7
   -parafilm or cover with septa
   -shake and vortex

10. Standard for intracellular fraction

    baba (5 nmol/mL)
    Phe (5 nmol/mL)
    2 % TCA
    saturated potassium borate buffer
    HPLC grade water

-combine in ashed HPLC vials in proportions of
1:1:2:4:5
-parafilm or cover with septa
-shake and vortex

11. Standard for protein bound Phe

To make amino acid standard solution:

100 µL baba (10 µmol/mL)
100 µL Phe (10 µmol/mL)
1 mL 2 % TCA
-combine in a test tube
-vortex

To make amino acid standard for injection:

amino acid standard solution
saturated potassium borate buffer
HPLC grade water

-combine in proportions of 1:100:550 in ashed HPLC vials
-parafilm or cover with septa
-shake and vortex

12. Sample for incubation medium

baba (5 nmol/mL)
sample
saturated potassium borate buffer
HPLC grade water

-combine in ashed HPLC vials in proportions of
1:2:2:8
-parafilm or cover with septa
-shake and vortex

13. Sample for intracellular fraction

baba (5 nmol/mL)
sample (5 nmol/mL)
saturated potassium borate buffer
HPLC grade water

-combine in ashed HPLC vials in proportions of
1:2:4:6
-parafilm or cover with septa
-shake and vortex

14. Sample for protein bound Phe

To make sample amino acid solution:

-add 200 µL of baba (10 µmol/mL) to EDL hydrolysate

-add 100 μL of baba (10 μmol/mL) to Epi hydrolysate
-shake and vortex

To make sample for injection:

amino acid solution
saturated potassium borate buffer
HPLC grade water

-combine in proportions of 1:100:550 in ashed HPLC vials
-parafilm or cover with septa
-shake and vortex

**Procedures**

The HPLC consisted of a Varian liquid chromatograph coupled to a fluorochrome detector. The fluorimeter was set at an excitation range of 340-380 nm and an emission cutoff at 390 nm. Phe and baba were derivatized with OPA immediately before the sample was injected onto the column. A C-18 reverse-phase column (Supelco) (75 x 4.6 mm I.D.; 40-60 μm particle size) was used for all samples. Two solvents, solvent A (see Appendix 5) and solvent B, methanol, were used to form the following gradient: 35% B at 0 minutes to 35% B at 9 minutes, 80 % B at 9.1 minutes to 10 minutes, and 35% B at 11 minutes to 12.5 minutes. This gradient was designed to increase the separation between Phe and valine, because of the large amount of valine (up to 1mM), which elutes near Phe.

Each sample was done twice. When the percent difference between duplicates was greater than 5%, it was repeated.

4. **Calculations**

When there were no treatment differences in Phe content of the intracellular fractions, protein degradation was based on the Phe content of incubation medium only. Total protein degradation was determined from the total amount of Phe released into the incubation medium and expressed as μg Phe released x $muscle^{-1}$ x 3 $hours^{-1}$. The fractional rate of protein degradation was calculated by dividing total protein degradation by the Phe mass, and was expressed as %/day.

REFERENCES

Tischler, M. E., M. Desautels, and A. L. Goldberg. Does leucine, leucyl tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? J. Biol. Chem. 257: 1613-1621, 1982.